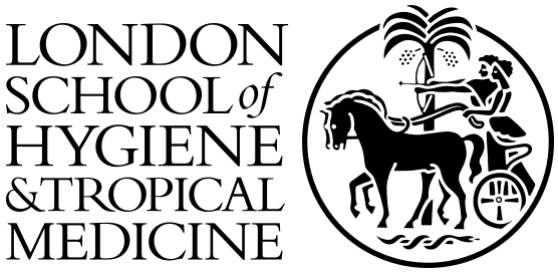

The impact of cytomegalovirus infection on natural killer cell responses to vaccines

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Declaration

I, **Carolyn M. Nielsen**, confirm that the work presented in this thesis is my own. Where information has been derived from other sources I confirm that this has been indicated in the thesis.

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Date: _____

Abstract

Vaccines are one of the most effective public health interventions, but factors influencing vaccine efficacy remain poorly understood. Natural killer (NK) cells contribute to adaptive immune responses following activation by IL-2 from memory T cells or crosslinking of CD16 by antigen-antibody complexes. Human cytomegalovirus (HCMV), a highly prevalent herpes virus, drives expansion of a mature CD56dimCD57+NKG2C+ NK cell subset, skewing the NK cell repertoire towards contact-dependent activation at the expense of cytokine sensitivity. I hypothesised that HCMV seropositivity would be associated with diminished NK cell activation during recall responses to vaccine antigens.

To test this hypothesis, I first confirmed my ability to detect NK cell responses following re-stimulation with vaccine antigens and described differential activation by CD57-defined NK cell subsets: mature CD56dimCD57+ NK cells produced less IFN-γ than CD56bright or CD56dimCD57- NK cells, consistent with their reduced responsiveness to IL-2. Next, in a cross-sectional study of 152 UK adults (36% HCMV+), I found that NK cell IFN-γ and degranulation responses to pertussis or H1N1 influenza vaccines were lower among HCMV+ individuals as compared to HCMV- individuals. The higher proportion of CD56dimCD57+NKG2C+ NK cells in HCMV+ individuals did not fully explain these impaired responses, as cells from all CD57/NKG2C-defined subsets responded less well. Finally, as I had detected lower expression of IL-18Rα on NK cells in HCMV+ individuals, I characterised pro-inflammatory cytokine interactions driving early NK cell activation and identified a central role for IL-18, due to its ability to synergise with IgG-CD16 crosslinking and common γ chain cytokines, including IL-2.

This work demonstrates, for the first time, that HCMV serostatus influences heterogeneity in NK cell contributions to adaptive immunity and raises important questions regarding the

impact of HCMV infection on vaccine efficacy. Furthermore, my work highlights that HCMV infection status is a major confounder of any study of human NK cell phenotype or function.

Acknowledgements

There are many people I would like to thank for their guidance and support over the last three years. Firstly, thank you to Eleanor for supervising my PhD project and for pointing me towards the MRC Vaccine Research Studentships when I approached you about PhD supervision four years ago. The MRC programme has played such an important role during my time here at LSHTM and has given me access to networking opportunities and funding that I wouldn't have otherwise benefited from. Thank you also for your encouragement and input at all stages of my PhD, including my GSK Vaccines placement and postdoctoral Fellowship applications. I've very much enjoyed being part of your research group and have particularly valued learning from you during the scientific writing process.

Thank you also to Matt White and Martin Goodier for your endless patience, feedback, and all of the lab technique and FlowJo training. One of my favourite parts of my PhD has been our discussions in the office and it really has been inspiring to work with people so excited about the science. Your day-to-day feedback has been invaluable. Thank you as well to Asia Wolf for keeping me company for all three years, for splitting the unsociable shifts of the cytokine experiments, and baking cakes when things seemed too serious. I hope the Biscuit Society continues in our absence.

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was therefore shared between the two of us and Ana Rodríguez-Galán. Martin also performed the influenza IL-2 blockades/ IgG-depletion assays and ELISAs with Chiara Lusa. In Chapter 5, the majority of the work was performed in conjunction with Asia Wolf.

For guidance with specific experiments, I'd particularly like to thank Brian de Souza for teaching me the protocols for plasma dialysis and IgG depletion, Tom Hall and Rebecca Dabbs for showing me how to set up in-house ELISAs, Adriana Goncalves for optimising the *NKG2C* PCR, and Steve Smith for giving advice on ELISPOTs. For superb lab management, thank you to Carolynne Stanley and Liz King for always answering questions, and to Carolynne for blood donor recruitment and Liz for flow cytometry technical support. Thank you also to Julius Hafalla for lending fresh eyes to my data every lab meeting and Christian Bottomley for helping me learn the statistical methods I needed to extend my analyses.

Finally, many thanks and love to my partner Tom and my family, who knew I wanted to do a PhD long before I did. This process has been all the more rewarding because of your support and faith in me. I hope you are looking forward to the natural killer cells quiz at Christmas.

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Chapter 1

Introduction

In this introductory chapter, I will start with a brief overview of the immune system and compare responses during primary and secondary pathogen exposure, before focusing in greater detail on natural killer (NK) cells. This will include an outline of the main characteristics of NK cells, the mechanisms and outcomes of NK cell activation, and the role of NK cells in adaptive immunity – particularly utilising the evidence from vaccination studies. I will then go on to discuss the epidemiology and health outcomes associated with human cytomegalovirus (HCMV) infection, with a focus on the impact of HCMV infection on cellular immunology and vaccine efficacy. Finally, I will summarise the main aims and research objectives of this PhD project.

1.1 The human immune system

The immune system is classically considered to be composed of the innate and the adaptive arms, each constituted of different types of leucocytes. For the innate system, this includes phagocytic cells and professional antigen-presenting cells (APCs) such as dendritic cells, monocytes and macrophages, as well as neutrophils, basophils, eosinophils, and NK cells. The adaptive side, by contrast, is comprised of only two major types of lymphocytes – B cell and T cells – though these can each be broken down into multiple, functionally distinct, subsets.

The key differentiating feature between these innate and adaptive leucocytes is related to the capacity to generate immunological memory. Through use of genetic recombination to produce a vast array of cell surface antigen-specific receptors, T cells and B cells are able to recognise an infinite number of conformational protein structures (B cells, via B cell receptor [BCR]) or pathogen epitopes (T cells, via T cell receptor [TCR]); for stimulation through the TCR, this involves antigen-specific recognition of pathogen-derived peptides presented by self Major Histocompatibility Class (MHC) Class I or II molecules, encoded in humans by Human Leucocyte Antigen (HLA) genes. Antigen-specific cells then rapidly clonally expand the antigen-specific population, before contracting to maintain a lower number of memory cells in

peripheral circulation. In contrast, innate immune cells do not have the capacity to genetically rearrange receptors and are not thought to form long-lived memory cells. Differentiation of T cells from naïve to central memory to effector memory phenotypes is discussed in section 1.4.1 (*HCMV impact on T cell repertoire)*.

1.1.1 Primary infections

Infection by a pathogen will be detected by the innate immune system, relying largely on recognition of conserved pathogen associated molecular patterns (PAMPs) by germline encoded Toll-like receptors (TLRs) or other pattern recognition receptors (PRRs). Activation of these innate cells drives production of a range of soluble mediators, such as cytokines and chemokines, driving inflammation through recruitment and activation of other immune cells. Moreover, the specific combinations of cytokines produced will strongly influence the quality of the subsequent immune response. There are multiple mechanisms through which innate cells may clear an infection, including engulfment and lysis of the pathogen through phagocytosis, direct killing of infected cells, or neutrophil extracellular traps which can kill bacteria extracellularly.

If the innate immune system does not swiftly control the infection, the antigen load will increase and after several days the immune response will expand to include the adaptive arm. In the absence of primed, pre-existing memory T cells or B cells, the adaptive response is slow as antigen-specific naïve cells are very low frequency and must clonally expand to reach sufficient numbers for an effector response. Trafficking of antigen by dendritic cells and other APCs into lymph nodes may also delay clonal expansion of the effector cells.

1.1.2 Secondary infections: the recall response

The dogma for the kinetics of a secondary infection is that the response by innate cells will be essentially the same as during primary exposure, due to the lack of true immunological

memory by these cells. In contrast, the adaptive arm is now primed to respond more quickly and more robustly due to increased numbers of circulating differentiated, memory, antigen-specific populations of both T cells and B cells. Additionally, antibodies specific to the pathogen will also be circulating in the plasma, capable of neutralising pathogens by marking them for opsonisation. Control of the infection should thus be faster during secondary exposure due to the increased frequencies of these primed and differentiated memory cells.

Indeed, the purpose of vaccination is to make use of this phenomenon and to introduce the immune system to non-virulent forms of pathogens, in order to generate immunological memory without the disease symptoms associated with primary infection. Protection therefore relies on the capacity of these immune cells to subsequently orchestrate an effective recall response to the pathogen upon a secondary exposure.

1.2 Natural killer (NK) cells

NK cells develop in the bone marrow from common lymphoid progenitor cells and constitute 5-20% of circulating leucocytes. While originally discovered in the context of their cytotoxic activity against tumour cells [3-6], NK cells are also important for early responses to infections (reviewed in [7]). In humans, NK cells are generally defined as innate CD3-CD56+ large granular lymphocytes: CD3 is the T cell co-receptor and not expressed on non-T cells, while CD56 (also known as Neural Cell Adhesion Molecule 1, NCAM1) is expressed on human NK cells but is of unknown function. CD56bright and CD56dim populations are functionally distinct, particularly in terms of ability to produce and be activated by cytokines [8]. CD56bright cells are generally more responsive to cytokine stimulation and robustly produce cytokines themselves, while CD56dim NK cells – representing 90% of all peripheral NK cells – are more mature, cytotoxic effectors and respond more robustly to direct stimulation [9], though still constitute the major proportion of cytokine-producing NK cells.

NK cells can be further divided into subsets based on expression of other cell surface markers, such as CD57 and CD16 [10-12]. CD57, a terminally sulphated carbohydrate epitope, remains of unknown function but acquisition is associated with maturation of NK cells and increasing functional skewing towards cytotoxic capacity rather than cytokine production [10,11]. Surface expression of CD16, a low affinity Fc receptor, is also associated with CD57 and a predisposition for direct contact activation [10,11]; indeed, CD56dimCD57+CD16+ NK cells are specialised for antibody-dependent cellular cytotoxicity (see section *1.2.1 Contact-dependent NK cell activation*, [11]). Increased proportions of CD57+ NK cells are associated both with ageing and with persistent human cytomegalovirus (HCMV) infection, as reviewed by myself and colleagues ([13], Appendix I).

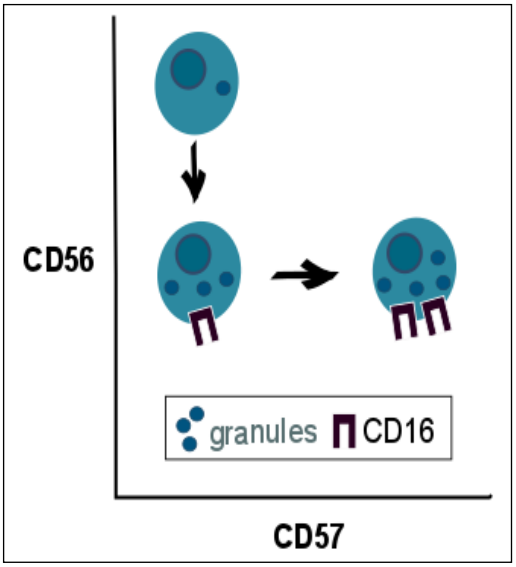

Figure 1. NK cell maturation from CD56bright to CD56dimCD57- to CD56dim CD57+. CD56bright NK cells have a high proliferative capacity and are very responsive to cytokine stimulation, while CD56dim NK cells are more sensitive to direct contact activation, e.g. through CD16, and have an enhanced cytolytic capacity due to the increased presence of pre-formed cytotoxic granules.

NK cell responses include both cytotoxicity and also secretion of cytokines and chemokines, including IFN-γ. The production of IFN-γ, which is essential for control of viruses and intracellular bacteria, is one of the major functional outputs of NK cell activation. IFN-γ has wide-ranging effects in the context of infection including: polarisation of T cell responses towards at Th1 phenotype; potent activation of monocytes/macrophages to increase anti-pathogen activities, antigen presentation and phagocytosis; and, contribution to driving B cell class switching to IgG isotypes effective for opsonisation [14-19]. Perhaps unsurprisingly, given this vast range of activities, over 300 genes are estimated to be upregulated following IFN-γ signalling [20]. Natural killer T cells and T cells also produce IFN-γ (indeed IFN-γ is a

characteristic of the Th1 response [17]) but the significance of the NK cell response lies partially in the rapidity of the production, which plays a key role in early control of infections (see section *1.1.3 NK cell contributions to adaptive immunity,* [7]), and also in that a large percentage of NK cells can respond to any given pathogen in contrast to the much lower frequency of antigen-specific T cells.

Cytotoxic NK cell responses, also referred to as cytolytic activity, involve killing of infected target cells or abnormal self cells. This is mediated through the secretion of lytic granules containing perforin, which punctures cell surface plasma membranes, and granzymes, which induce cell death through apoptosis (reviewed in [21]). These granules traffic to the cell surface membrane at the immunological synapse along microtubule filaments, orchestrated by the microtubule organising centre which has migrated to the synapse [21]. Deposition of CD107a (also known as LAMP-1) from these granules on the plasma membrane during fusion at the synapse can be used as a proxy measurement of degranulation and cytotoxic activity (discussed in more detail in Chapter 3 [22,23]). It is interesting to note that these perforin and granzyme pathways mediating cytotoxicity are conserved between NK cells and T cells, despite obvious differences in mechanisms of activation [21]. Specifically, in contrast to T cell stimulation via the TCR, activation of NK cells may be initiated, without prior sensitisation, by direct binding of activating receptors overcoming inhibitory signals, or by exogenous cytokines, as detailed below.

1.2.1 Contact-dependent NK cell activation

Following the discovery of NK cells as autonomous killers of tumour cells in the 1970s [5], it was observed that detection of transformed cells was associated with down-regulation of MHC Class I molecules [24]. This led to the development of the 'missing self hypothesis', which describes the phenomenon whereby NK cells identify and kill somatic cells with lowered expression of 'self' MHC Class I molecules [25].

Direct, contact-dependent, stimulation of NK cells has therefore historically been considered the classical route of activation. NK cells vary at the single cell level in their expression of specific germline-encoded inhibitory and activating receptors (within and between individuals), and activation depends on the balance of engagement of these receptors (reviewed in [26]). In the missing self model, for example, it is the lack of an inhibitory signal (in the absence of ligation of an NK cell inhibitory receptor by MHC Class I molecules) that permits NK cell activation [25].

The range of NK cell activatory and inhibitory receptors is staggering in its complexity, with one family in particular, KIRs (killer immunoglobulin-like receptors) showing extensive polygenic and polymorphic diversity (reviewed in [27]). KIRs are one of three major inhibitory receptor families that bind MHC Class I molecules, alongside immunoglobulin-like transcripts (ILTs), and CD94/NKG2 heterodimers (e.g. CD94/NKG2A, which recognises HLA-E). All of these inhibitory receptors contain an immunoreceptor tyrosine-based inhibition motif (ITIM) in the cytoplasmic tail which initiates signalling cascades during engagement of the receptor (reviewed in [28]). Engagement of inhibitory receptors on NK cells with self MHC molecules also serves the purpose of NK cell licensing (also known as education), which must occur before NK cells can be fully activated by other stimuli (reviewed in [29]).

A minority of KIR and NKG2 molecules have an immunoreceptor tyrosine-based activatory motif (ITAM) on their cytoplasmic tails instead and function as activating receptors [30,31]. Of particular interest in the context of this thesis is the CD94/NKG2C heterodimer, which also recognises HLA-E as its ligand, discussed in detail below (see section *1.3.2 HCMV impact on NK cell repertoire*). Other activatory receptors include 2B4, DNAM-1, NKG2D, and the natural cytotoxicity receptors (NCRs) NKp80, NKp30, NKp44 and NKp46 [32]. NKG2D, another member of the NKG2 family, recognises UL-16 binding proteins (ULBP) induced by DNA damage, and also MHC Class I chain-related proteins A/B (MICA/B) [33]. Ligands for NCRs are unknown in

many cases but include the tumour protein B7-H6 (NKp30 [34]), influenza haemagglutinin antigen (NKp46 [35]), and the poxvirus haemagglutinin (both NKp30 and NKp46 [36]). 2B4 and DNAM-1 bind CD48 and CD155/ nectin-2 as their natural ligands respectively.

Also of relevance is the low affinity Fc receptor (FcRIII), CD16, which can be crosslinked by antigen-bound IgG, such as in immune complexes or antibody-coated infected cells, driving a process called antibody-dependent cellular cytotoxicity (ADCC) as well as the production of cytokines [37,38]. CD16 is a unique activating receptor in that its ligation can trigger activation of NK cells in the absence of other co-stimulation [39]. This represents an important interface of the innate and adaptive arms of the immune system (detailed further in section *1.1.3 NK cell contributions to adaptive immunity).*

Finally, accessory cells can also contribute to NK cell activation through contact-dependent mechanisms, for example through ligation of LFA-1 by ICAM-1 [40]. In the context of infection, upregulation of NK cell activating receptor ligands by accessory cells can function as sufficient co-stimulation to over-ride inhibitory signals provided by MHC Class I competent cells (reviewed in [2], see Figure 2 and section *1.2.2 Cytokine-mediated NK cell activation*, below).

1.2.2 Cytokine-mediated NK cell activation

Alongside these direct routes of activation, it is also well-established that NK cells can be activated indirectly by soluble signalling molecules and indeed it has become increasingly clear that NK cells are often not fully functional during pathogen responses until triggered by pro-inflammatory cytokines, released by accessory cells (reviewed in [2], see Figure 2). This does not contradict our classical understanding of NK cells as innate lymphocytes — that do not require co-stimulation or prior exposure to antigen to become activated — but, rather, indicates synergies between multiple pathways.

PRRs in accessory cells such as dendritic cells, monocytes, and macrophages detect PAMPs very early in infection and upregulate co-stimulatory molecules and begin pro-inflammatory cytokine production, which act to recruit and activate NK cells [41,42]. Strong, contact-independent soluble signals can over-ride the inhibitory contact-dependent signals from MHC Class I molecules, as signalling through soluble ligands is not subject to inhibition through MHC I [26], and lead to NK cell activation in the form of cytokine production or cytotoxicity.

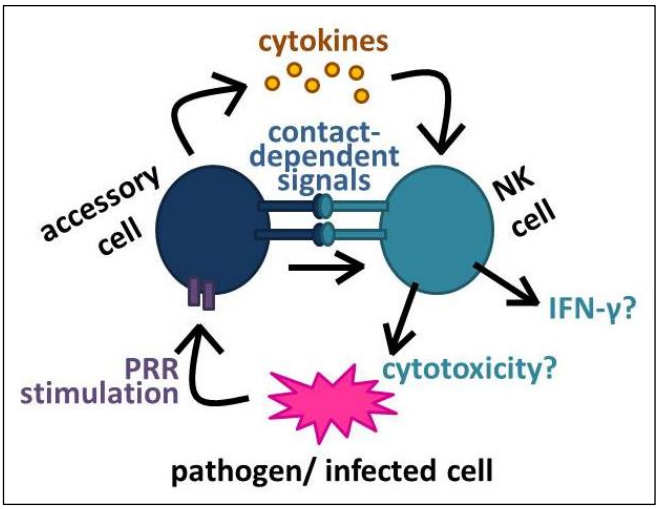

Figure 2. NK cell activation by accessory cells in response to infection. Accessory cells respond to pathogens following stimulation of pathogen recognition receptors (PRRs), such as TLRs. Accessory cells can then activate NK cells through secretion of soluble signals, including cytokines, alongside direct contact activation involving ligation of NK cell activating receptors. NK cells may then go on to produce inflammatory cytokines themselves, such as IFN-γ, or perform cytolytic killing. Adapted from [2].

The main pro-inflammatory cytokines produced by accessory cells that can drive NK cell activation are IL-12, IL-18, and IL-15. Type I interferons (IFN-α and IFNβ) are also produced swiftly during infection and are another major early cytokine activator of NK cells, particularly during viral infections, and are associated with NK cell cytotoxicity rather than IFN-γ production [43]. This thesis does not focus on the role of type I IFNs in NK cell activation, although a study by our group does include a brief exploration of the role of IFN-α in the context of influenza vaccination (Goodier, Lusa, Rodríguez-Galán, Nielsen, *et al.*, manuscript accepted) and further information on type I IFN activation of NK cells can be found in multiple reviews [2,7].

IL-12 is produced primarily by monocytes and dendritic cells following PRR stimulation, or signals from activated T cells and NK cells. In the NK cell field, IL-12 is best recognised for its

role as a powerful driver of IFN-γ production by NK cells, but IL-12 also skews antigen-specific T cells towards a Th1 phenotype (including IFN-γ production), and activated B cells towards production of Ig isotypes associated with Th1 responses and control of infections (reviewed in [44]). Similarly, IL-18 is secreted by myeloid cells, particularly macrophages, after inflammasome stimulation [45] and also plays a role in NK cell IFN-γ responses by synergising with IL-12. Indeed, IL-18 was initially identified as an 'IFN-γ-inducing factor' [46]. IFN-γ can act on myeloid cells to induce IL-12 and IL-18 production, thus generating a positive pro-inflammatory feedback loop [17].

IL-15 has many cellular sources but is also produced predominantly by monocytes and dendritic cells in response to activation with stimuli such as type I interferons or TLR engagement. One of the mechanisms of action of IL-15 is to enhance cytotoxicity by inducing the upregulation of integrin LFA-1 during development, which enables adhesion to target cells through its ligand ICAM-1, thus stabilising the immune synapse [47]. Due to its essential role in NK cell development, maturation, and survival [48,49], IL-15 is required for long-term cell cultures to provide a survival signal [50,51].

IL-2 is also a member of the common γ chain cytokine family (which includes IL-4, IL-7, IL-9, IL-15, and IL-21) which share the common γ chain component of their respective cytokine receptors (reviewed in [52]). IL-2 shares many structural and signalling similarities to IL-15 and the two cytokines have some overlapping functions with respect to NK cell activation, particularly as related to driving proliferation. The additional role of IL-2, as produced by CD4+ T cells upon TCR engagement, is discussed below (see section *1.1.3. NK cell contributions to adaptive immunity*) and cytokine receptor expression is a key focus of Chapter 5.

1.2.3 NK cell contributions to adaptive immunity

While NK cells have traditionally been classified as cells exclusively of the innate immune system, recent research has implicated them as potential mediators of adaptive responses through their activation by adaptive T cell responses, specifically vaccine antigen-specific CD4+ T cell-derived IL-2 [53-57]. The heightened IFN-γ response of NK cells in the context of a vaccine recall response suggest that NK cells may play a role in protection from vaccine-preventable diseases, particularly as NK cells respond more quickly than T cells upon re-exposure to vaccine antigens [54]. Dissecting the role of NK cells, both as a whole population and by subset, in different vaccination models is of interest in order to understand the overall contribution of NK cells to protective immunity post-immunisation.

It is well established that different subsets within CD4+ and CD8+ T cells respond differently during responses to vaccine antigens, e.g. naïve vs. central memory subsets, and furthermore that long-term changes to the T cell repertoire may affect the responsiveness of this arm of the adaptive immune response (reviewed in [58]). However, the possibility that different NK cell subsets also respond differently to vaccine antigen stimulation has not been explored, despite evidence that responsiveness to cytokines decreases with CD57 expression [10]. As with T cells, factors that influence the relative proportions of NK cell subsets will likely also influence the capacity of the entire NK cell compartment to respond to vaccine antigens, e.g. ageing or certain infections.

There is substantial evidence from numerous studies that CD4+ T-cell-derived IL-2 can activate NK cells, which can then contribute to the recall response, predominantly through production of IFN-γ. The first indication of this phenomenon in humans came from work by Fehniger *et al* who showed that CD56bright NK cells could be activated by T cell IL-2 in the lymph node, calling this 'a potential new link between adaptive and innate immunity' [59]. Later work by He *et al* demonstrated that NK cells produced IFN-γ when stimulated with influenza A (*n* = 26

[53]). Similar results were achieved using live or heat-inactivated whole virus. He *et al* went on to reveal, albeit with small sample sizes and no statistical analyses, that this NK cell IFN-γ response was dependent on the presence of T cells, as shown by an abrogation of the response with CD3-depletions (*n* = 8), thus removing T cells, and that it could be substantially suppressed in peripheral blood mononuclear cell (PBMC) cultures by a blocking antibody to IL-2 (*n* = 6), or rescued in a CD3-depletion cultures by addition of exogenous IL-2 (*n* = 3). In addition, it was confirmed that T cell production of IL-2 in response to influenza A stimulation preceded the NK cell IFN-γ production, though only published for one subject.

Long *et al* subsequently showed in a vaccine intervention study that NK cells are significant producers of IFN-γ *in vitro* following influenza vaccination; seven of eight vaccinees had an increase in IFN-γ-producing NK cells post-vaccination, defined as greater than two standard deviations from the pre-vaccination mean [55]. An enhanced post-vaccination response was seen only to thimerosal-inactivated whole influenza A virus, not to haemagglutinin and M1 peptide pools. Furthermore, as no increased IFN-γ production was observed with CD4+ T cells and only two subjects had enhanced CD8+ T cell responses, the authors went on to show that the NK cells represented a major effector population during these re-stimulation responses. The proportion of the IFN-γ-producing cells that were NK cells increased from a mean of 16.4% pre-vaccination to 30.3% post-vaccination. To note, however, even including only the peak post-vaccination responses for each subject, the percentage of NK cells producing IFN-γ was still very low (0.05-2.00% for subjects considered to be 'noteworthy' responders).

Previous work from our group built on this early work with a rabies vaccination study, demonstrating conclusively that enhanced IFN-γ and degranulation NK cell responses during re-stimulation with inactivated rabies virus, 21 days post-vaccination, were dependent on IL-2 from CD45RO+ CD4+ T cells which peaked after six hours (*n* = 5, [54]). The effect was entirely dependent on T cell memory, as pre-vaccination NK cells were comparably activated when

cultured with post-vaccination T cells. This study also determined that IL-2 was necessary but not sufficient, as the NK cell responses to rabies virus were additionally dependent on IL-12 and IL-18 from accessory cells. Of particular interest from this work was the novel appreciation of the extent of the NK cell contribution to these 'recall' responses: NK cells comprised over 70% of IFN-γ-producing or degranulating cells in the first 18 hours. Even after seven days, the proportion of IFN-γ+ cells that were NK cells remained high at 30-50%. This finding underscored the key role NK cells play early in infection, prior to control and clearance by the adaptive immune system. This study also demonstrated that IL-2 drove NK cell proliferation, resulting in successive waves of NK cell responses.

However, responding to IL-2 from T cells is not the only mechanism by which NK cells may be activated by the adaptive immune system during a memory response. As mentioned above (see Figure 3 and section *1.1.1 Contact-dependent NK cell activation*), CD56dim NK cells express the low affinity IgG receptor CD16 at the cell surface. CD16 recognises the Fc portion of IgG, and thus NK cells can be activated by crosslinking of CD16 receptors through ligation by IgG molecules either bound to a pathogen, forming an immune complex, or on the surface of an infected cell. This CD16 crosslinking induces a signalling transduction pathway, leading to cytotoxicity — antibody-dependent cellular cytotoxicity (ADCC) — and cytokine production, including IFN-γ and TNF-α [37]. This signalling is mediated by intracellular adaptor proteins associated with CD16, CD3ζ or Fcε receptor I γ (FcεRIγ) [60,61], and involves a rapid increase in calcium ions (Ca^{2+}), required for IFN-γ production in response to CD16 stimulation, and a subsequent signalling cascade with similarities to that of the T cell receptor [37,62,63].

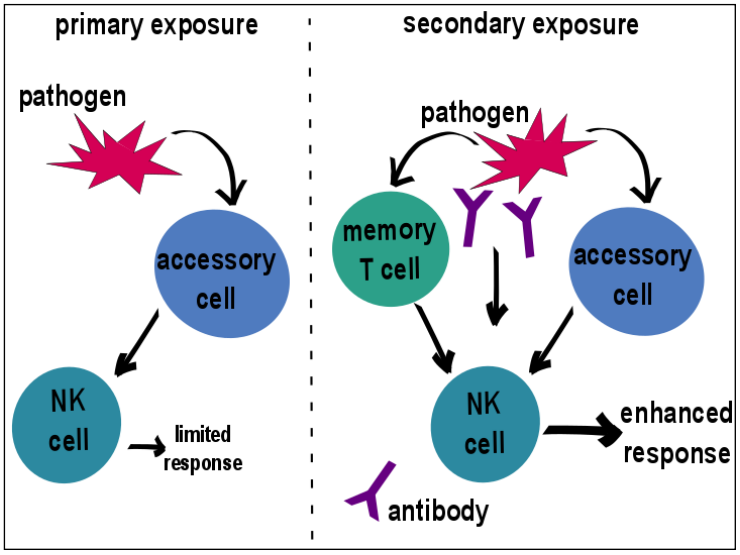

Figure 3. NK cell activation in adaptive immunity. NK cells can be activated by accessory cytokines alone (see Figure 2), but during secondary recall responses, NK cell responses can be further potentiated by IL-2 from antigen-specific CD4+ T cells, and also by antibody-antigen complexes via Fc receptors on NK cell surfaces, e.g. CD16. This leads to enhanced activation.

HIV vaccine studies have indicated antibody-driven immunity can be mediated through NK cells, and that ADCC activity by autologous NK cells is correlated with the quantity of IgG1 bound to HIV-infected cells [64]. As reviewed by Kramski *et al* [65], HIV-specific antibodies capable of mediating ADCC have been associated with a range of positive outcomes including better disease prognosis, such as slower progression of infection. While the majority of evidence for a post-vaccination ADCC role for NK cells comes from these HIV trials, there is also consistent data from the influenza field showing, in mice, that ADCC mediates protection from disease at low challenge doses following vaccination with a conserved M2 protein vaccine, and that this was dependent on NK cells, but not complement [66].

It is also possible for NK cells to be activated during vaccination through signals from other compartments of the innate immune system, as well as those from the adaptive arm. For example, Neves *et al* detected a correlation between upregulation of CD69 and TLR-3/ TLR-9 in NK cells following immunisation with the yellow fever vaccine (n = 8 [67]). Given the concurrent increase in plasma IFN-γ, they posited that TLRs may play a key role in NK cell activation during vaccination and NK cell contribution towards the establishment of immune memory. While NK cells were not confirmed to be the cellular source of this augmented IFN-γ, and CD69 and TLR expression are not functional read-outs in themselves, this data is

consistent with the hypothesis that NK cells can be activated following immunisation with a range of vaccines and are an interesting example that in some instances NK cells may be directly activated by pathogens.

To note, alongside this gradual increase in our understanding of NK cells as effectors of secondary immune response through signals from the adaptive immune system, there have also been high-profile publications reporting 'true' NK cell memory, i.e. long-lived, antigen-specific, expanded NK cells that have a heightened response upon secondary exposure. The concept of NK cell immunological memory in humans remains contentious [68], but there are data from three different contexts suggesting this area warrants further work: cytokine-induced memory-like NK cells (not antigen-specific), murine cytomegalovirus-induced NK memory (antigen-specific), and liver-restricted NK cell memory, e.g. to haptens (antigen-specific; as reviewed in [39,69]). The latter two scenarios are yet to be demonstrated in humans, but several groups have shown cytokine-induced memory-like cells in humans ([70,71], reviewed in [72]). Here, NK cells stimulated with IL-12, IL-15 and IL-18, then washed and cultured for three weeks, show higher IFN-γ upon re-stimulation. Given the significance of cytokine stimulation in NK cell responses to vaccine antigens, this is of particular interest in the context of this thesis work and indeed we have observed a similar cytokine-induced phenomenon in the context of influenza vaccination (Goodier, Lusa, Rodríguez-Galán, Nielsen, *et al*., manuscript accepted). A detailed discussion of this particular study is outside the scope of this thesis, but pertinent aspects are covered in Chapters 4-5.

1.3 Human cytomegalovirus (HCMV)

HCMV is the largest member of the herpesvirus family, with a double-stranded DNA genome of 236kb in wild type strains [73]. HCMV is also known as herpesvirus-5 (HHV-5) and is part of the herpesvirus B subclass, which includes other herpesviruses that infect lymphocytes i.e. lymphotrophic virus (HHV-6) and HHV-7. Other well-known herpesviruses belong to subclass A,

which infect neurons (herpes simplex-1 [HSV-1], herpes-simplex-2 [HSV-1], and varicella zoster [VZV]), or subclass C, which also infect lymphocytes but additionally can cause lymphoproliferation (Epstein-Barr virus [EBV], and Kaposi's sarcoma-associated herpesvirus [KSHV, or HHV-8]).

As a herpesvirus, which has co-evolved with humans for millions of years [74], HCMV causes persistent infections and is extremely well-adapted to the human host. The molecular mechanisms for maintaining chronic and latent states remain poorly understood [75].

1.3.1 HCMV epidemiology

Transmission of HCMV can occur via infected body fluids (including saliva, breast milk, or urine), or through blood transfusion and organ transplants [76]. Congenital infections can also take place during pregnancy, potentially through infection of trophoblasts progenitor cells, although the mechanism is unclear [77]. In developing countries, such as the Gambia, prevalence of HCMV is high with the majority of infants infected in the first year of life [78]. In contrast, the prevalence of HCMV infection in more developed countries is lower initially, though associated with socioeconomic status, and increases with age [79]. In the United Kingdom, 15% of 1-4-year old children are infected, rising to 30% by age 20-29, and to approximately 80% by 65-years [80]. Infections in childhood are predominantly via breast milk [81] during infancy or, later, through child-to-child transmission, e.g. in nurseries [82].

During primary infection, HCMV-infected cells can be detected in mucosal tissues including the cervix, salivary glands, breast, or intestine. From here, following an initial lytic cycle, HCMV can spread into adjacent underlying tissues but also, importantly, into bodily secretions, perpetuating transmission. HCMV spread within the body is both through free virus particles in the blood, and through cellular trafficking. As reviewed by Sissons *et al* [83] and Stevenson *et al* [84], alongside haematopoetic stem cells, monocytes are key host cells of HCMV during

latency [85,86]. While monocytes are not permissive to viral replication themselves, HCMV infection of monocytes can drive myeloid cell differentiation to macrophages [87], which do support the full HCMV life cycle. Indeed, infected macrophages can be detected in HCMV-seropositive individuals [88]. HCMV may also infect and be carried by epithelial and endothelial cells [89].

Following primary infections, HCMV virions are shed for a substantial period of time before latency is established. In adults, viral shedding may persist for several months [90], whilst in children this may last several years (as reviewed in [91]). It is therefore unsurprising that one of the strongest predictors for seroconversion in an adult is having a shedding child (reviewed in [91]). Reactivation is also associated with a return of viral shedding [92], though in healthy adults this will ultimately be controlled once again through largely T cell and NK cell mechanisms, with potentially some assistance from antibody (reviewed in [93]). Reactivation is a normal part of the viral life cycle, which may be precipitated by stress or other disruption to the host immune system.

1.3.2 Health outcomes in HCMV infections

HCMV infections in adults are normally latent and asymptomatic, unless the individual is immunosuppressed [94]. As the immune system can effectively control latent infections, where cells may be infected but the virus has very low levels of replication or gene expression, there may be few or no clinical symptoms. Conversely, 10% of congenital infections cause 'CMV disease' with symptoms such as jaundice, hepatomegaly, and neurological defects present from birth [76,95,96]. Indeed, HCMV remains the leading cause of non-hereditary childhood hearing loss or mental retardation [76,97]. The risk of transmission to the foetus is greatest when primary infection of the mother occurs during pregnancy; this results in congenital infection in 40% of cases [98]. Reactivation of latent infections during pregnancy is also associated with increased odds of infection *in utero*, albeit with much decreased risk of

sequelae [99]. Similarly, in the absence of antiviral treatment, HCMV can cause severe pathology during reactivation when hosts are immunocompromised [100].

However, there is strong evidence to suggest that even in asymptomatic individuals HCMV infection has an effect on long-term health outcomes; HCMV has been linked with several chronic diseases including cardiovascular disease [101-103], cancers [104,105], vascular dementia [106,107] as well as functional impairment or frailty in the elderly [108-110]. Multiple studies have examined the relationship between HCMV infection and overall mortality, although usually in specific subsets of national populations [110-114]. An exception is a study using the recent US NHANES III (National Health and Nutrition Survey, 1988-1994) which included a nationally representative sample of 33,994 adults, aged 25-years and older [115]. Analysis of data from a 10-year follow-up of this cohort demonstrated that HCMV infection was associated with a significantly increased risk for all-cause mortality (Hazards Ratio: 1.19, 95% CI: 1.01, 1.41 [103]). Furthermore, high anti-HCMV antibody titres, not just HCMV seropositivity, have also been strongly correlated with frailty, functional impairment of the immune response, all-cause mortality, and cardiovascular mortality [102,110,114,116-119]. There has also been a report from Zambia that HCMV infection during infancy can negatively impact early childhood growth, development, and overall health [120], though it is not certain how generalisable this is to other countries.

It is therefore clear that HCMV infection negatively impacts a variety of measures of health outcomes. To understand the mechanisms behind this phenomenon it is useful to evaluate the components of the immune system that HCMV infection affects.

1.4 Intersection of HCMV and immunology

At the cellular level, HCMV infection has an impact on both T cell and NK cell populations. The initial immune response during acute infection directs these long-term effects as the

responding subsets of cells undergo sustained maturation and expansion; indeed, HCMV infection drives one of the largest cellular immune responses in humans [121]. In contrast, little is known about the effect of chronic HCMV infection on B cell populations.

1.4.1 HCMV impact on T cell repertoire

In HCMV-seropositive (HCMV+) individuals, a very large proportion of both CD4+ and CD8+ memory T cells is directed against HCMV [121]. Van de Berg *et al.* demonstrated that within the CD4+ T cell compartment there is an accumulation of CD28- T cells which are HCMV-specific as they proliferate and produce IFN-γ in response to HCMV antigen [122]. This work corroborates earlier studies that identified the CD28- phenotype as the most common for CD4+ T cells in HCMV+ donors [123, 124]. CD28, the CD80 and CD86 ligand, is the co-stimulatory receptor required for signalling through the TCR and is central to IL-2 production [125]. Absence of CD28 expression could potentially result in reduced T cell activity and, particularly relevant for this project, reduced IL-2 production could putatively diminish the NK cell response to vaccine antigens. However, this would only be true if HCMV infection affected the entire T cell repertoire in this manner, not just the HCMV-specific populations. In reality, it is not entirely clear to what extent HCMV infection impacts the development and maintenance of CD4+ or CD8+ T cell responses to other antigens (discussed in more detail in Chapter 6).

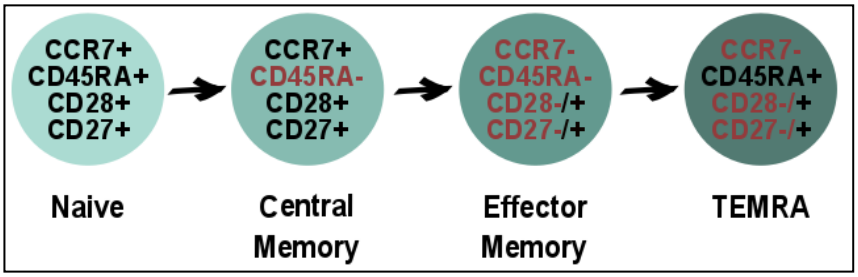

Figure 4. Phenotypic changes associated with T cell maturation. The markers CCR7 (a chemokine receptor), CD45RA (a protein tyrosine phosphatase isoform), CD28 (the CD80 and CD86 ligand), and CD27 (a co-stimulating receptor of the TNF family) can be used to define four main T cell differentiation subsets: naïve, central memory, effector memory and terminally differentiated effector memory (TEMRA) cells [1].

In terms of the CD8+ T cell compartment, a review of nine papers in 2003 suggested that the dominant phenotype for T cells in the latency or memory stage of a HCMV infection was CCR7-CD27-CD28-CD45RA+ as the presence of this subset correlates with HCMV seropositivity but not with previous exposure to other viruses including measles, EBV or VZV [126,127]. A more recent study looked specifically at CD27-CD45RA+ CD8+ T cells, and indeed found that accumulation of this subset appears specific for HCMV as no association was seen with HIV, hepatitis C, EBV or herpes simplex virus (HSV) infections [122]. The expansion of this differentiated — and potentially exhausted — subset alongside a simultaneous reduction in the relative number of naïve T cells has implicated HCMV as a driver of age-associated immunological changes [128]. Indeed, the gradual loss of CD28, CD27, CD45RA and CCR7 on large proportions of T cells seen in chronic HCMV infections is classically used to define the maturation/ exhaustion process for both CD4+ and CD8+ T cells (see Figure 4, [1]).

The impact of these shifts towards mature T cell phenotypes, including in the context of vaccine responses, has not been resolved but it has been suggested by several groups to be indicative of a degree of immunosenescence (e.g. [103,129-134]). The number of functional HCMV-specific CD8+ T cells is similar in young and old donors, but the phenotypes of these cells differ with the age group [135]; cells from older donors express more KLRG1 (an inhibitory receptor), while cells from younger donors express more CD28 [135]. There therefore appears to be a simultaneous effect of age, or perhaps duration of HCMV infection, alongside HCMV serostatus itself. However, it is interesting to also note that recent work has demonstrated that HCMV-specific T cells remain functional in the elderly and there is no evidence of telomere erosion, which would be indicative of terminal differentiation and exhaustion [136]. Additionally, the rarity of HCMV disease from reactivation or *de novo* infection in elderly populations strongly suggests that anti-HCMV immunity functions remarkably well in these populations, despite indicators of immunosenescence [137].

1.4.2 HCMV impact on NK cell repertoire

HCMV infection is also known to drive changes in the NK cell population. In particular, HCMV infection is strongly associated with expansion of expansion of NKG2C+ NK cells [138-140]. While the murine cytomegalovirus (MCMV) peptide m157 directly binds NK cells via Ly49H and can induce proliferation and activation of this Ly49+ subset, an equivalent molecular interaction has yet to be as clearly identified in humans; although CD94/NKG2C can bind the HCMV peptide UL18 with low affinity, UL18 is not required for the NKG2C+ NK cell expansion [141]. More recently, it was demonstrated that the mechanism for HCMV-infected fibroblast-driven expansion of NKG2C+ NK cells *in vitro* was dependent on CD14+ monoycte-derived IL-12, as well as the interaction of NKG2C with its HLA-E ligand on HCMV-infected cells, stabilised by the UL40 HCMV peptide [142].

As noted previously, NKG2C is a C-type lectin activating receptor that forms heterodimers with CD94 at the NK cell surface to recognise its ligand HLA-E. NKG2A, an inhibitory receptor, also forms heterodimers with CD94 and binds HLA-E. Although NKG2C and NKG2A can be co-expressed, HCMV-driven expansion of NKG2C+ NK cells is generally associated with loss of NKG2A [138,143]. In addition to being NKG2A-, NKG2C+ NK cells driven by HCMV infection express lower levels of NCRs including NKp30 and NKp46, and are more likely to be KIR+ than NKG2A+ NK cells [138]. The expansion of these NKG2C+ NK cells expressing self-specific KIRs results in a permanent change in the type and frequency of KIR+ NK cells [139,144]. Other groups have shown that NKG2C+ NK cells preferentially acquire CD57 [11,145]. Lopez-Vergès *et al.* demonstrated that the preferential expansion of NKG2C+CD57+ NK cells in HCMV+ patients was achieved first by NKG2C+ NK cells proliferating, then becoming NKG2Chi, and then by finally acquiring CD57 [140]. NKG2C+ NK cells degranulate and secrete cytokines such as IFN-γ and TNF-α in response to direct contact stimulation, e.g. crosslinking antibodies or HLA-E binding, but do not respond well to IL-12 and IL-18 stimulation [139,146].

It has therefore been hypothesised that HCMV shapes the NK cell repertoire in otherwise healthy individuals in such a way as to potentially affect their ability to control future infections, particularly in the context of cytokine stimulation [138,145]. Both CD57+ and NKG2C+ expression are associated with a reduced ability to respond to cytokine stimulation but, to our knowledge, the relative contributions of these two markers to this functional profile have not yet been dissected, e.g. do NKG2C-CD57+ NK cells respond to IL-12 and IL-18 better than NKG2C+CD57+ NK cells? Understanding this potential synergy may help illuminate the relative importance of HCMV infection (driving expansion of CD57+NKG2C+) and ageing (driving CD57+ expansion alone) in shaping NK cell responses.

1.4.3 Associations between HCMV serostatus and vaccine responses

The work discussed above suggests that HCMV may drive the differentiation of NK cell subsets that are less functional during vaccine responses, i.e. less responsive to antigen-specific CD4+ T cell IL-2. However, these studies have generally been done in the context of organ transplants, while studies on the effect of HCMV infection on immune responses to vaccination have thus far been broadly restricted to work in two populations: elderly European cohorts receiving seasonal vaccination against influenza, and infants in the Gambia receiving the routine measles childhood vaccine. In both cases, analysis focused on antibody and T cell responses and there was no evaluation of NK cells.

With respect to the effect of HCMV in the elderly demographic, there is contradictory evidence from different studies. Trzonkowski *et al.* demonstrated an association between HCMV infection and lower antibody titres against influenza haemagglutinin post-immunisation in elderly care-home residents (aged 65-99 years; *n* = 91 [119]). Indeed, there was a strong reciprocal correlation between anti-HCMV and anti-haemagglutinin antibody titres, showing the humoral response to the influenza vaccine was lower in those with HCMV infection, especially in those with high anti-HCMV antibody titres. Similarly Moro-García *et al* detected a

negative correlation between anti-influenza and anti-HCMV antibody titres, with the titres adjusted for time since immunisation (*n* = 100 [117]). Derhovanessian *et al* also reported an association between HCMV seropositivity and lower humoral responses in adults >60-years old, though not in younger adults (*n* = 30 [147]).

In contrast, den Elzen *et al.* observed no difference between the anti-haemagglutinin responses of HCMV- and HCMV+ care home residents after seasonal influenza vaccination [148]. To note, den Elzen *et al.* only analysed the response to H3N2, which is the haemagglutinin antigen with which Trzonkowsk*i et al.* saw the weakest association [119,148]. However, supporting the conclusions of den Elzen *et al*, Wald *et al* similarly detected no significant difference in seroconversion rate or titre in a haemagglutinin inhibition assay to the pandemic 2009 H1N1 influenza vaccine between HCMV- and HCMV+ adults aged above 65-years (*n* = 55 [149]). Similarly, O'Connor *et al.* saw no effect of HCMV serostatus on IgG response to pneumococcal vaccination in 50-70-year old adults (*n* = 331 [150]).

Further studies are certainly needed to better understand this interaction of HCMV infection and the immune response to influenza vaccination, however, as McElhaney *et al.* observed in their 2012 review, post-vaccination antibody titres alone are generally not sufficient to predict vaccine failure in adults [151,152]. It is therefore important to include analyses of cellular responses when evaluating the impact of HCMV on vaccine immunogenicity, particularly given our knowledge of the effect of HCMV chronic infections on NK and T cell repertoires. Similarly, HCMV serostatus is a crude measure and HCMV reactivation/ shedding may be a more biologically relevant parameter for characterising HCMV infection.

Cellular responses were briefly addressed in the first paper of the Gambian infant studies. In 2008, Miles *et al.* demonstrated that the absolute number of naïve undifferentiated T cells (CCR7+CD27+CD28+) in peripheral blood remained constant with HCMV infection while there

was a large increase in differentiated CD8+ T cells (CD28-CD57+) and smaller increase in CD4+ T cells as compared to blood samples obtained prior to HCMV infection [153]. Despite this, there was no difference in cytokine production or proliferative response of T cells to re-stimulation with measles vaccine antigen between HCMV-/+ infants. In fact, antibody titres against measles correlated with the IFN-γ response to HCMV, suggesting HCMV infection may in fact be enhancing the humoral response to the measles vaccine. The authors thus concluded that HCMV infection induces T cell differentiation without impairing antigen-specific responses, which would be consistent with the changes to the T cell repertoire being limited to the HCMV-specific cells. McElhaney *et al.* indeed comment in their review that a direct link is yet to be established between changes in CD8+ T cell populations by HCMV infection and vaccine failure [152]. However, duration of HCMV infection was one year at most in this study, and we know ageing and possibly duration of HCMV infection can also affect responses [119,135].

The next paper, in 2010, went on to examine the effect of HCMV infection on responses to a polysaccharide vaccine in addition to measles, and also looked at the impact of EBV infection [154]. They observed no difference in antibody response between HCMV-/+ infants [154]. However, infection with EBV did result in a reduced antibody response to the measles vaccine antigen and, interestingly, co-infection with HCMV seemed to rescue this response [154]. As the previous paper had shown that HCMV drives CD4+ T cell differentiation, it was hypothesised that HCMV infection may enhance anti-measles antibody production through non-specific upregulation of CD4+ T cell-mediated help [153,154]. The authors therefore once again concluded that HCMV did not affect vaccine antibody responses, but conceded that the interaction of HCMV infection and vaccination needs to be studied on a larger scale [154].

The two populations discussed above — the elderly and infants — represent demographic extremes, and the robustness of recall responses to vaccine antigens are clearly relevant for

public health in healthy adults as well. Bosch *et al.* reported a lower response to influenza vaccination in HCMV+ university students (Third International Workshop on CMV and Immunosenescence [155]), but at the time of commencing this doctoral work, there was limited published literature in young adults. Specifically, Trzonkowski *et al* found significantly higher titres of anti-HCMV IgG in non-responders to the influenza vaccine (aged 19-40 years; *n* = 63) while Wald *et al* had demonstrated higher anti-haemagglutinin titres in HCMV- subjects (aged 18-64 years; *n* = 52). Over the last two years, more studies have been published that include younger vaccinees [156,157]; the significance of these publications is discussed in the final chapter of this thesis.

More importantly perhaps for the justification of this study, there as yet has been no research — in any age group — on the relative NK cell responses to vaccine antigens in HCMV-/+ individuals. As with T cells, the significance of the NK cell phenotypes driven by HCMV infection are poorly understood, but it is hypothesised that they may affect the ability of NK cells to respond to vaccine antigens during a recall response, or during initial vaccination. Specifically, with respect to CD57+NKG2C+ NK cells, these cells may not lose the ability to mediate ADCC in response to crosslinking by vaccine antigen-specific IgG, which binds CD16, but their responses to cytokines produced by antigen-specific CD4+ T cells and activated APCs, such as macrophages, may be reduced.

1.5 Aims and research objectives

Although the data are clear on the effect of HCMV on NK cell phenotype, there remain many gaps in our knowledge as to the functional consequences of such NK cell changes, particularly in the context of vaccination and infection. Given the evidence cited above, my central hypothesis was that I would observe an increased proportion of CD56dimCD57+NKG2C+ NK cells in HCMV+ individuals and, due to the decreased responsiveness of these cells to exogenous cytokine stimulation, I would detect reduced NK cell activation to vaccine stimulation in the HCMV+ group. Conversely, I expected to see a retained capacity to mediate ADCC in the HCMV+ individuals, as there is no evidence to suggest the HCMV-driven CD56dimCD57+NKG2C+ subset should respond less robustly to CD16 crosslinking.

The overall aim of this PhD project was therefore to investigate the effect of HCMV serostatus on NK cell responses upon re-exposure to antigens from previously administered vaccines.

The main research objectives were to:

a) Determine whether NK cell response to vaccines are determined by their maturation status, as defined by CD56/CD57 expression (**Chapter 3**)
b) Characterise and compare the response of NK cells to stimulation with previously encountered vaccines between HCMV- and HCMV+ individuals (**Chapter 4**)
c) Identify the key synergies between innate and adaptive signals early in NK cell activation that may influence the magnitude of these NK cells responses (**Chapter 5**)

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Chapter 2

Methods

For completeness, all methods corresponding to the data presented in this thesis are listed here. Methods used in more than one chapter are described in detail, while protocols exclusive to individual chapters have been cross-referenced to the respective chapters', more relevant, Methods sections [1-3].

2.1 Preparation of buffers

Table I describes the buffers prepared for use in the experiments outlined below.

Table I. Preparation of buffers.

Buffer	Composition	Relevant Experiments
freezing medium	80% FBS* (Gibco) 20% DMSO-Hybri-Max™ (Sigma)	PBMC cryopreservation
FACS buffer	PBS (Gibco) 5mM EDTA (Invitrogen) 0.05% sodium azide (Sigma) 1% FBS* (Gibco)	flow cytometry
MACS buffer	PBS (Gibco) 0.5% FBS (Gibco) 5mM EDTA (Invitrogen)	CD4+ T cell depletion
coating buffer	1.59g Na_2CO_3 2.93g $NaHCO_3$ made up to one litre with distilled water, pH 9.3-9.7	Total IgG and anti-pertussis toxin (PT) IgG ELISAs

* foetal bovine serum

2.2 Isolation of PBMC

Up to 50ml of venous blood was collected in 50ml tubes (CellStar) containing 100µl heparin (1000IU/ml; Wockhardt). Heparanised blood was carefully layered onto Histopaque (Sigma) at a 2:1 ratio then spun at 524g for 30 minutes, without the use of the centrifuge brake. PBMC were then collected from the buffy coat layer which forms at the plasma and histopaque interface using 3ml transfer pipettes (Fisher Scientific). PBMC were washed twice in complete medium (RPMI 1640 supplemented with 100IU/ml penicillin/streptomycin and 20mM L-glutamine [Gibco, Lifesciences]) by spinning at 754g for 10 minutes. PBMC were then filtered through a 70µm cell strainer (BD Falconer; or EASYstrainer™ Cell Strainer, Greiner Bio-One) to

exclude cell clumps and counted using trypan blue (Sigma) exclusion at a 1:1 dilution on a haematocytometer (FastRead slides, Immune Systems). PBMC were resuspended at $2x10^7$ cells/ml in complete medium.

2.3 Cryopreservation of PBMC

PBMC at $2x10^7$ cells/ml were aliquoted into 500µl cryotubes aliquots (Thermo Scientific) then mixed with 500µl chilled freezing medium (Table I). The PBMC cryovials were placed immediately in 100% isopropanol baths (Mr Frosty™, Thermo Scientific) and kept at -80°C at least overnight until transfer to liquid nitrogen for longer term storage.

Prior to use, PBMC were thawed in complete medium pre-warmed to 37°C, washed, and rested in complete medium supplemented with 10% AB plasma (Sigma) for at least 30 minutes before use. Thawing of cells was performed by quickly pipette mixing the pre-warmed medium into the cryopreserved cells and transferring to 50ml tubes containing 25ml pre-warmed medium. This rapidly diluted the DMSO as cells thawed to prevent toxicity. Cells waiting to be thawed were kept on dry ice and only removed from liquid nitrogen storage on the day of use.

For some assays, where 10% AB plasma was not used in cell culture, PBMC were resuspended in medium supplemented with FBS, autologous plasma, or IgG-depleted AB plasma (Chapter 4), as appropriate. Preparation of these alternatives is described in individual chapters as relevant. To note, all FCS, AB and autologous plasma used in *in vitro* assays were heat-inactivated either by the manufacturer (FCS), or in-house by incubation at 56°C for 30 minutes (AB and autologous).

2.4 Cell culture

All cell culture experiments were performed in a total volume of 100µl/well. Culture conditions are described in detail in individual chapters. Complete cell culture medium was supplemented 10% pooled AB plasma unless otherwise stated.

2.4.1 Standard 18 hour *in vitro* assay

The main *in vitro* assay used in this thesis work involved culture of approximately $2x10^5$ PBMC/well in 96-well U-bottom plates (Nunc) in complete medium supplemented with 10% AB plasma for 18 hours in 5% CO_2 at 37°C. A high concentration of cytokines IL-12 (5ng/ml) and IL-18 (50ng/ml) was routinely used as a positive control (see Chapter 3 for validation work). For assays where upregulation of CD107a was a read-out, 2 µl/well sterile anti-CD107a antibody (A488-conjugated; BD Biosciences) was included for the entirety of cell culture. When IFN-γ production was a read-out, GolgiStop (containing Monensin, 1/1500 concentration; BD Biosciences) and GolgiPlug (containing brefeldin A, 1/1000 concentration; BD Biosciences) were added three hours before the end of cell culture, i.e. after 15 hours.

2.4.2 Crosslinking assays

For activation via crosslinking of NK cell surface receptors, 96-well flat-bottom plates (Nunc) were coated with 20µg/ml antibody to NK cell receptors, e.g. anti-human CD16 (BD Biosciences), or isotype-matched control antibodies, e.g. mIgG1$_k$ (BD Biosciences), overnight at 4°C. Wells were rinsed with PBS before addition of approximately $4×10^5$ PBMCs/well. In some instances these cells had had been incubated overnight at 37°C in 10% AB plasma, with or without IL-2, in 5ml polypropylene round-bottomed tubes (Falcon). Two µl/well sterile anti–CD107a-A488 antibody was always added at the beginning of culture, and all assays were performed in complete medium supplemented with 10% AB plasma. Cells were not routinely

assessed for IFN-γ production but, in these instances, GolgiStop and GologiPlug were used as described above.

Cells were harvested after five or 18 hours by transfer to 96-well U-bottom plate. The 96-well flat-bottom plate used for crosslinking was then incubated at 37°C for a further 15 minutes with 100µl/well pre-warmed PBS (5mM EDTA) to remove remaining any plate-bound cells. After vigorous pipette mixing, this volume was then transferred to the 96-well U-bottom plate.

2.5 Flow cytometry

Prior to staining of cells with fluorophore-conjugated antibodies for flow cytometry, 96-well U-bottom plates were centrifuged at 754g for five minutes, either directly from the incubator (e.g. standard 18 hour assay), or after a two-step transfer from flat-bottom to U-bottom plates (i.e. all crosslinking assays). Supernatants were then flicked off before vortexing the plates to resuspend the cells. Cells were washed with 200µl/well FACS buffer (Table I) and centrifuged at 754g for five minutes. FACS buffer was then flicked off and the plate was vortexed. The cells were then stained with fluorophore-conjugated antibodies to cell surface markers (10µl master mix/ well) for 20 minutes in the dark at 4°C, then washed again with 200µl FACS buffer as described above, and fixed with 75µl/well Cytofix/Cytoperm (BD Biosciences) for 30 minutes in the dark at room temperature. Cells were washed with 175µl/well Perm/Wash (diluted 1:10 in de-ionised water; BD Biosciences) and centrifuged at 931g for five minutes, then Perm/Wash was flicked off and the plate was vortexed. Cells were stained for intracellular molecules (10µl master mix/ well) for 15 minutes in the dark at room temperature, then washed once more in FACS buffer as described above but centrifuging at 931g. Cells were then transferred from the plate to microtubes (Alpha Laboratories) by vigorously pipette-mixing 100µl of FACS buffer in the wells, twice. Microtubes, containing samples suspended in 200µl FACS buffer, were placed in 5ml polystyrene round-bottomed tubes (Falcon) for acquisition on an LSRII flow cytometer (BD Biosciences) using FACSDiva® software.

Compensation controls were prepared at the same time as cell staining to accurately model any antibody decay or bleaching between staining and acquisition. Either Compbeads (BD; anti-mouse IgG_K and negative control from the same lot) or OneComp (eBioscience) beads were used for compensation controls and were vortexed in 500µl FACS buffer. One µl of antibody was added to 50µl bead solution in 5ml polystyrene tubes (Falcon) and incubated for 15 minutes at room temperature in the dark. Controls were washed by adding 500µl FACS buffer per tube and centrifuging at 754g for five minutes. FACS buffer was then discarded and 400µl fresh FACS buffer was added to the beads. Compensation controls were stored with the samples in the dark at 4°C until acquisition on the LSRII flow cytometer (BD Biosciences), a maximum of three days after staining.

Data analysis was performed using FlowJo V10 (Tree Star). FACS gates to define cell populations (e.g. NK cells) were applied in a standard format, and then adjusted per sample as necessary. FACS gates to measure responses (e.g. CD107a+) were set on unstimulated cells (medium alone or isotype controls) and were applied in standard format across all conditions per donor. Gating strategies were developed during preliminary experiments, as detailed in Chapter 3.

2.6 Enzyme-linked immunosorbent assays (ELISAs)

ELISAs were used to determine the HCMV serostatus of donors (Chapters 4 and 6), to confirm depletion of IgG from plasma passed over a protein G Sepharose column (Chapter 4), and to calculate anti-H1N1 and anti-pertussis toxin (PT) IgG titres in autologous plasma (Chapter 4). Detailed methods for these ELISAs are described in Chapter 4, (section 4.2.3), as is the method used for IgG depletion of AB plasma for cell culture (section 4.2.2).

2.7 ELISPOTs

HCMV- and HCMV+ donor IL-2 responses to pertussis were compared using an IL-2 ELISPOT (Mabtech) as outlined in Chapter 4, section 4.2.4.

2.8 *NKG2C* genotyping

Donor were genotyped for *NKG2C* by PCR to allow comparison of NK cell responses between *NKG2C* $^{-/-}$, *NKG2C* $^{-/+}$ and *NKG2C* $^{+/+}$ HCMV- or HCMV+ donors. Details of this PCR are given in Chapter 4, section 4.2.5.

2.9 Statistical analyses

Statistical analyses were performed in Prism 6 (GraphPad Software) or STATA (Stata/IC 14) as detailed in individual chapters. For flow cytometry analyses, samples where the cell population being analysed had fewer than 100 events were excluded. For trend analyses, all samples from a given donor were excluded if any contained fewer than 100 events. Sample sizes and number of experiments represented in each figure are described in legends.

Statistical tests were routinely two-sided (exceptions to this are noted in individual chapters' methods sections, e.g. Chapter 4) and nonparametric tests were always used for samples where $n \leq 30$.

2.10 References

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Chapter 3

Differential activation of CD57-defined natural killer cell subsets during recall responses to vaccine antigens

The work presented in this chapter is adapted and extended from White*, Nielsen* *et al* 2014 ([1], Appendix II).

3.1 Introduction

Natural killer (NK) cells are classically regarded as a stable population of innate immune effectors that, by cytokine production or cytotoxicity, target tumour cells or help to contain infection until an effective adaptive response is mounted. However, evidence now suggests that NK cells can exhibit augmented responses in the context of secondary pathogen exposure, despite their lack of germline encoded receptors with which to generate true antigen-specific memory.

These enhanced NK cell responses therefore rely on signals from the adaptive immune system and there are two main routes by which this NK cell activation occurs: stimulation by antigen-specific T cell-derived IL-2, or crosslinking of the low affinity Fc receptor, CD16, by antigen-antibody (IgG) complexes. IL-2 secreted by memory CD4+ T cells promotes NK cell function and proliferation, while CD16 crosslinking initiates the process of antibody dependent cellular cytotoxicity (ADCC) [2-6]. IL-2 and IgG do not act on NK cells in isolation, but rather in the context of the ongoing inflammatory response following detection of infection by the innate arm of the immune system, such as production of pro-inflammatory cytokines.

The NK cell repertoire in an individual comprises a heterogeneous group of cells (see Chapter 1) and thus there is substantial variation in their capacity to respond to both cytokine signalling and direct contact activation, such as CD16 crosslinking. A number of NK cell subsets with different functional potential have now been described in humans. The least mature circulating NK cells are CD56brightCD57− and are assumed to give rise to CD56dimCD57- cells which, in turn, mature

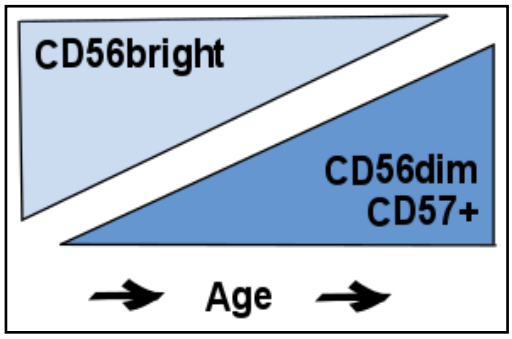

Figure 5. Changes in the NK cell repertoire associated with ageing. During healthy ageing there is a gradual accumulation of mature CD56dimCD57+ NK cells and proportional decrease in immature CD56bright NK cells.

into CD56dimCD57+ cells, the latter subset increasing in frequency with increasing age (Figure 5 [7,8]). Despite the well-described associations with NK cell maturation, CD57 itself, a terminally-sulphated carbohydrate epitope (glucuronic acid-3-sulphate), remains of unknown function or molecular location on NK cells [8-11]. Regardless, there are many clear demonstrations of associations between CD57 expression on NK cells and ageing or various disease states, as reviewed by myself and colleagues [8].

The three-step maturation (from CD56bright, to CD56dimCD57- to CD56dimCD57+) is associated with acquisition of CD16, CX3CR1 (a chemokine receptor), granzyme and KIR, gradual loss of proliferative capacity, reduced responsiveness to cytokines such as IL-12 and IL-18, and increasing cytotoxic function [12,13]. CD56dimCD57+ NK cells express lower levels of IL-18Rα [13] as well as lower levels of mRNA for the inducible chain of the IL-12R (IL-12Rβ2) [14] suggesting that these NK cells may respond less well than other subsets to IL-12 and IL-18 due to decreased expression of the respective receptors. Conversely, CD56dimCD57+ cells express higher levels of CD16, explaining why they are particularly good mediators of ADCC [14].

The potential for NK cells to respond to exogenous cytokines is central to their ability to control infections [4,15,16], particularly where ligands for other NK cell activating receptors are lacking. Moreover, NK cells responding to CD4+ T-cell-derived IL-2 have the potential to contribute to secondary immune responses, including those induced by vaccination [4,5]. I wondered, therefore, whether NK cell subsets would differ in their ability to mount 'recall' responses to vaccine antigens. If so, this would have consequences for the ability of NK cells to contribute to secondary responses in any individual where the distribution of NK cells is skewed towards the CD56dimCD57+ phenotype, such as in the elderly or cytomegalovirus infected (HCMV+) individuals, as explored in Chapter 4.

To test this hypothesis, I have assessed the capacity of various NK cell subsets, defined principally by their expression of CD56 and CD57, to contribute to recall responses to various vaccines, focusing on responses to components of the diphtheria–tetanus–pertussis (DTP) among previously-vaccinated adults.

3.2 Methods

3.2.1 Study subjects

For preliminary work (Section A of Results), volunteers were recruited from among staff and students at the LSHTM. All subjects gave fully informed, written consent under a protocol for recruitment of blood donors approved by the LSHTM ethics committee (reference # 5520, Appendix III) to provide ≤ 50ml venous blood. For the work adapted from White *et al*, 2014 (Section B of Results), subjects ranged in age from 21-73 years and all donors reported diphtheria, tetanus and pertussis vaccination status ($n \leq 33$). All subjects gave fully informed, written consent and the study was approved by the LSHTM Ethics Committee (reference # 6237, Appendix IV).

3.2.2 PBMC preparation and cell culture

Peripheral blood mononuclear cells (PBMC) were isolated, cryopreserved, and thawed as described in Chapter 2.

PBMC (approximately 2×10^5 cells in 200 µl) were cultured in 96-well U-bottom plates in complete medium (see Chapter 2) with or without low concentration of cytokines (LCC; 12·5 pg/ml recombinant human (rh) IL-12 [PeproTech] plus 10 ng/ml rhIL-18 [MBL]); high concentration of cytokines (HCC; 5 ng/ml rhIL-12 plus 50 ng/ml rhIL-18); or 7·5 µg/ml tetanus toxoid (NIBSC: 02/232, Appendix V), 1 µg/ml diphtheria toxoid (NIBSC: 69/017, Appendix VI), or 1 IU/ml killed whole cell pertussis (NIBSC: 88/522, Appendix VII) for 18 hours at 37°C. GolgiPlug and GolgiStop (BD Biosciences) were added as described in Chapter 2. Additional time series experiments were performed, without the addition of GolgiPlug and GolgiStop, harvesting cells between 4-18 hours, every two hours.

The following stimuli were also used in preliminary experiments at varying concentrations (see Table II): live attenuated measles (NIBSC: 92/648), live attenuated mumps (NIBSC: 90/534), live attenuated rubella (NIBSC: 91/668), live attenuated yellow fever (NIBSC: 99/616), inactivated whole virus hepatitis A (NIBSC: 95/500), inactivated whole virus rabies (NIBSC: 07/162), meningococcal C polysaccharide (NIBSC: 07/318), pertussis toxin (NIBSC: JNIH-5), and killed whole cell typhoid vaccines (NIBSC: TYVL).

Table II. Vaccines included in pilot studies. Vaccines selected for further studies are shaded grey. Note that for measles, mumps and rubella, IU refers to 'infectious units', whereas for hepatitis A and yellow fever, IU denotes 'international units'. The data sheet for rabies does not specify.

Type of Vaccine		**Pathogen**	**Working Concentration (see Figures 4-6)**		
			Low	**Medium**	**High**
virus	**whole-live**	measles	$4x10^2$ IU/ml	$8x10^2$ IU/ml	$1.6x10^3$ IU/ml
		mumps	$8x10^2$ IU/ml	$1.6x10^3$ IU/ml	$3.2x10^3$ IU/ml
		rubella	$1.6x10^2$ IU/ml	$3.2x10^2$ IU/ml	$6.4x10^2$ IU/ml
		yellow fever	$2x10^{2.5}$IU/ml	$4x10^{2.5}$IU/ml	$8x10^{2.5}$IU/ml
	whole-inactivated	hepatitis A	1IU/ml	2IU/ml	4IU/ml
		rabies*	0.0066IU/ml	0.033IU/ml	0.066IU/ml
		H1N1 influenza~	7.5µg/ml		
bacteria	**whole – killed**	typhoid ǂ	$1x10^5$ orgs	$5x10^5$ orgs	$1x10^6$ orgs
		pertussis	-	0.1IU/ml	1IU/ml
	subunit	meningococcal C (polysaccharide)	20µg/ml	40µg/ml	80µg/ml
		tetanus (toxoid)~	4µg/ml		
		diphtheria (toxoid)	5µg/ml	10µg/ml	20µg/ml
		pertussis (toxin)	2ng/ml	20ng/ml	100ng/ml

* Units refer to Pitman Moore rabies virus glycoprotein antigen content.
~ Previously titrated by other members of the group; titrations not shown.
ǂ Data from further studies not shown. Units refer to number of whole organisms (orgs) of *Salmonella enteric* subsp. *typhi*.

For receptor crosslinking experiments, assays were set up as outlined in Chapter 2 using mouse monoclonal antibodies to human CD16 (working concentration of 20 µg/ml; BD Biosciences), or a cocktail of monoclonal antibodies to human NK receptors ([NKG2D, NKp30, NKp46, 2B4 (all from R&D Systems)] and CD2 (BD Biosciences) at an overall combined concentration of 20 µg/ml, i.e. 4 µg/ml each], or an equivalent concentration of mouse IgG1 *κ* isotype control

antibody (BD Biosciences) as a negative control.

3.2.3 Flow cytometry

Responses of NK cells and T cells were assessed as described previously and outlined in Chapter 2 [17]. The following reagents were used: anti-CD56-phycoerythrin (PE)-Cy7, anti-CD16-allophycocyanin (APC)-H7, anti-IFN-*γ*-e780, anti-IFN-*γ*-APC, anti-CD3-V500 (all BD); anti-CD25-PE, anti-IL-18R*α*-PE, and anti-CD57-e450 (all e-Biosciences). Anti-IL-12Rβ2 monoclonal antibody was obtained from R&D Systems and conjugated to PE-Cy5 using an Easylink PE/Cy5® Conjugation Kit (Abcam). Compensation controls were prepared at the time of cell staining using OneComp beads (eBioscience).

3.2.4 Statistical analyses

Unless otherwise stated, figures in Section A show data from single experiments while figures from Section B show data from 18 experiments (2-3 donors per experiment); sample sizes and number of experiments represented in each figure are described in legends. Flow cytometry and statistical analyses were performed as described in Chapter 2 and also as detailed in figure legends. Individual gated cell populations were excluded from analyses if they contained fewer than 100 cells. $****p \leq 0.0001$, $***p < 0.001$, $**p < 0.01$, $*p < 0.05$.

3.3 Results

For clarity, this Chapter has been divided into two sections: unpublished preliminary work **(*Section A*)** and published work **(*Section B*)** adapted from White*, Nielsen* *et al* 2014 ([1], Appendix II).

Section A

3.3.1 Identifying the NK cell population

The first step of flow cytometry data analysis is to restrict analysis to single cells ('singlets') using the forward scatter height (FSC-H) against forward scatter area (FSC-A) to exclude events that are too large to be a single cell (Figure 6A). This is important to prevent confounding the analysis of specific cell subsets, as aggregates of cells will have markers for multiple different cell types or activation states. Following exclusion of such clumped cells, the next stage in analysis of NK cells within PBMC populations is to identify the lymphocytes. This gate is selected based on FSC-A and side scatter area (SSC-A), which indicate size and cell granularity (or internal complexity), respectively (Figure 6B). The FSC-A vs SSC-A gating also allows exclusion of dead cells as the change in morphology during cell death results in lower forward scatter and higher side scatter as compared to live cells, and thus different FSC-A and SSC-A profiles. While it was not possible to include a live/dead fluorophore-conjugated marker (which is detectable only in dead cells due to loss of membrane integrity leading to internalisation of the marker) as the fluorescent channel was already occupied by other antibodies, work by other members of my group had previously demonstrated that the above strategy was sufficient to exclude dead cells from the analyses.

In humans, NK cells are defined as CD3-CD56+ granular lymphocytes (Figure 6C). While expression of CD16 is also often used to identify the NK cell population, or subsets of NK cells, it is not a stable marker and is therefore not appropriate for use in *in vitro* assays involving NK

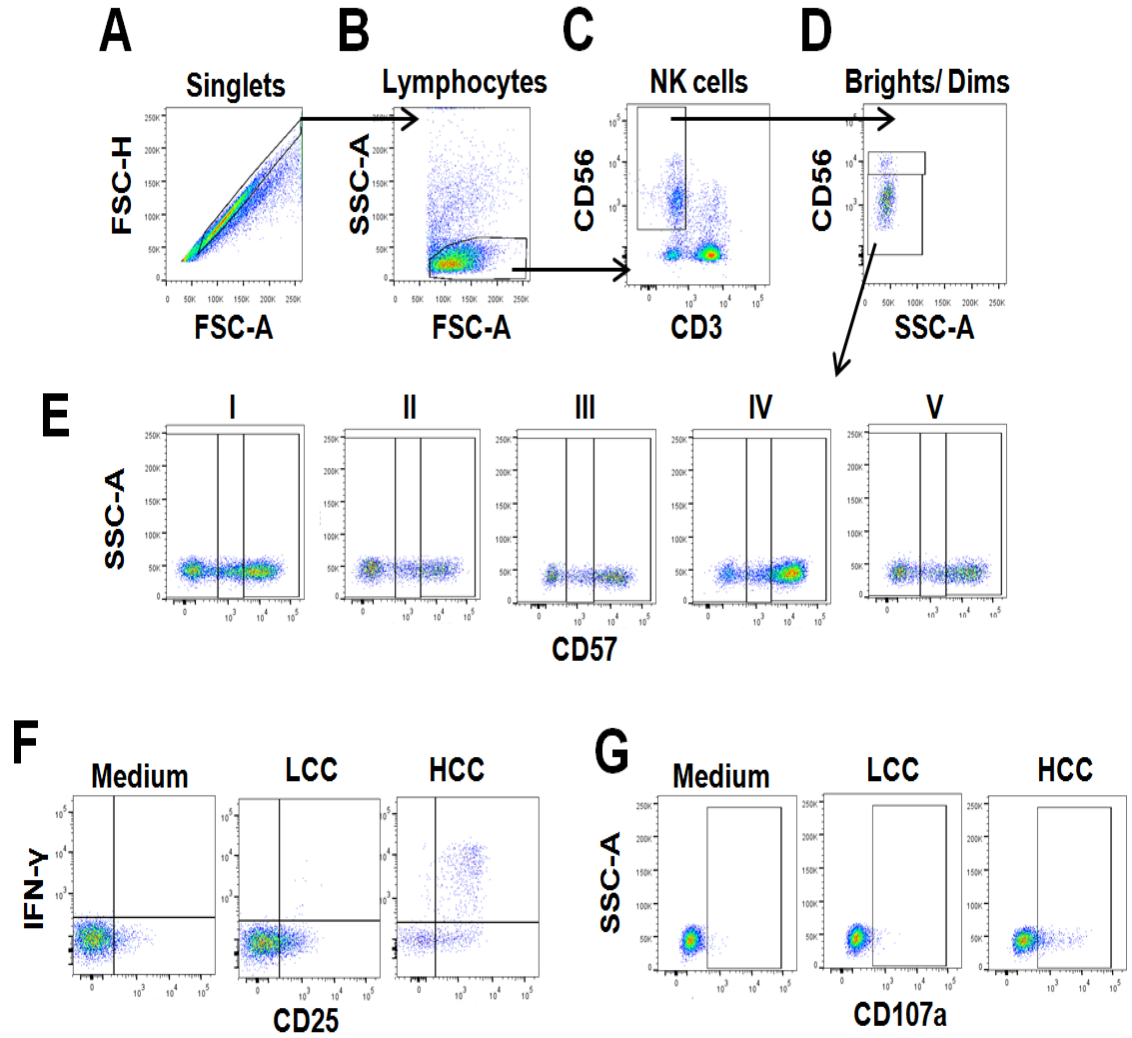

Figure 6. Gating strategy for analysing NK cells. PBMC were cultured for 18 hours with medium alone, a low concentration of cytokines (LCC; 12.5pg/ml IL-12 and 10ng/ml IL-18), and a high concentration of cytokines (HCC; 5ng/ml IL-12, 50ng/ml IL-18). The gating strategy for singlets **(A)**, lymphoyctes **(B)**, NK cells **(C)**, Brights/Dims **(D)** is shown for a single donor, while the gating of CD56dim NK cells into CD57- (left), CD57int (middle) and CD57+ (right) is shown for five representative donors (I-V). Functional gating is shown as upregulation of CD25/IFN-γ **(F)** and CD107a **(G)** when cultured with medium alone, LCC, or HCC. Cell clumps can be seen as dots outside the gate in A, while many of the ungated cells in B are likely dead cells.

cell stimulation; kinetics of CD16 expression in relation to NK cell activation are explored further in Chapter 5.

Finally, within the CD3-CD56+ NK cell population there are two main – functionally distinct – subsets: CD56brights and CD56dims. These two populations can be easily be differentiated during flow cytometry analysis based on CD56 expression (Figure 6D). The CD56dim population can then be further defined based on CD57 expression, which is a marker of unknown function associated with maturation. Previous work has generally split CD56dims into CD56dimCD57- and CD56dimCD57+ [12,14], but observation of the CD57 FACS profiles suggested to us that there may be an intermediate population (Figure 6E); this is one of the focuses of this chapter (specifically, see sections 3.3.6-3.3.7).

3.3.2 Improving sensitivity of NK cell activation read-outs

To measure NK cell responses *in vitro*, I am interested in upregulation of expression of CD25, IFN-γ, and CD107a.

CD25 is the high affinity receptor for IL-2 (IL-2Rα) and is expressed at negligible levels on resting NK cells. The other components of the IL-2R, the common γ chain (CD132) and β chain (CD122), are constitutively expressed. Upregulation of surface expression of CD25 therefore indicates both NK cell activation and sensitisation to IL-2 signalling. The synergies between IL-2 and the stimuli that upregulate CD25 are explored in detail in Chapter 5.

Production of IFN-γ is one of the key functions of NK cells early during infection due to its central role in guiding a Th1 response, activating phagocytes, and synergising with other pro-inflammatory cytokines. Measuring an NK cell IFN-γ response involves detecting an increase in intracellular IFN-γ, which accumulates prior to secretion. This necessitates fixing and permeabilising the PBMC population during staining (to allow intracellular access of

fluorescently-conjugated-anti-IFN-γ) and also inclusion of brefeldin A and monensin during cell culture to prevent trafficking of proteins from the endoplasmic reticulum to the Golgi, leading to a detectable accumulation of intracellular IFN-γ *in lieu* of secretion.

As previous work from our group had indicated IL-2 was essential for NK cell IFN-γ responses to vaccine antigens [4], I opted to identify activated NK cells as cells co-expressing CD25/IFN-γ, i.e. CD25+IFN-γ+ (Figure 6F, see also Figure 11). The rationale for this was that NK cells would only produce IFN-γ (i.e. IFN-γ+) in the presence of optimal IL-2 signalling, which requires expression of CD25 (i.e. CD25+). Any NK cell detected as CD25-IFN-γ+ was therefore likely background activation and defining IFN-γ-producing NK cells as CD25+IFN-γ+ would therefore improve my sensitivity. Furthermore, CD25 upregulation in itself is not a functional marker and thus I was less interested in CD25+IFN-γ- NK cells which, although activated, would not be truly (yet) responding to the *in vitro* stimulus.

For this reason, when selecting a low concentration of the pro-inflammatory cytokines IL-12 and IL-18 (LCC) to use in NK cell activation cultures, concentrations were chosen to be below those which are required to drive a CD25+IFN-γ+ response on their own (IL-12: 12.5pg/ml and IL-18: 10ng/ml; Figure 6F). Addition of LCC to culture is particularly useful when assessing NK cell activation to subunit vaccines, e.g. tetanus toxoid, which are purified proteins and do not contain the pathogen-associated molecular patterns (PAMPs) that would naturally stimulate accessory cells. LCC therefore mimics this production of IL-12 and IL-18 to co-stimulate NK cells. These concentrations were determined by titration prior to the commencement of this project, but further studies elucidating the mechanisms of interaction between IL-12, IL-18, IL-2 and other cytokines are the focus of Chapter 5, and the data presented therein confirm that these concentrations are appropriate.

Lastly, I am interested in NK cell cytotoxicity, involving release of granules containing perforin and granzymes. CD107a (LAMP-1) is an endosomal marker that fuses to the cell surface membrane during exocytosis and is an established proxy for measuring degranulation [18,19]. Inclusion of anti-CD107a in cell culture media allows capture of CD107a at the cell surface, preventing recycling, and thus measurement of the cumulative degranulation response over the entirety of cell culture [20]. Gating of CD107a+ NK cells is straightforward (Figure 6G).

To note, use of brefeldin/monensin blocks movement of vesicles to the cell surface and the timing of their addition to culture is therefore a compromise between capturing peak IFN-γ and maximal CD107a (degranulation) responses. For this reason, I habitually add brefeldin/ monensin three hours before the end of culture, i.e. after 15 hours in an 18 hours culture, or after three hours in a five hour culture. This strategy allows us to measure both the peak IFN-γ response (after 15-18 hours, see below) and the peak CD107a response (by 4-5 hours, see below). The selection of culture time points is discussed further below.

3.3.3 NK cell activation times series

An *in vitro* culture duration of 18 hours had previously been used in this lab and elsewhere for measuring NK cell responses to various stimuli. An earlier publication by our group had established that this was an optimal time point for measuring IFN-γ [4], but a thorough time series analysis of CD25 expression had not yet been performed. Due to my interest in the CD25+IFN-γ+ response, understanding the kinetics of CD25 expression following NK cell activation was also important.

PBMC were therefore stimulated with medium alone, or high concentrations of IL-12 and IL-18 (HCC) and harvested over an 18 hour time series. Surface expression of CD25 on the NK cell surface was measured at multiple time points, and also *ex vivo*. We observed culturing PBMC in medium alone was sufficient to upregulate CD25 to the surface of a small percentage of NK

cells, but that this remained low in the absence of further *in vitro* stimulation (Figure 7A). When cultured in the presence of HCC, a significant proportion of NK cells upregulated CD25 to the cell surface, which reached a peak after 16 hours. This gives us confidence that while the peak of CD25 upregulation in response to pro-inflammatory cytokine stimulation may not be at precisely 18 hours, expression is still high enough at this point to permit detection. Indeed, there was no significant difference between 16 and 18 hours (Wilcoxon signed rank test, p = 0.0625), although we should note this test is not powered to detect small differences given the interdonor variation and relatively small sample size (n = 5).

I am also interested in the kinetics of the NK cell CD107a response as a proxy for degranulation, particularly of relevance for later investigations into the ADCC pathways of NK cell activation by the adaptive immune response. Surface expression of CD107a was therefore also measured during this time series, by adding anti-CD107a to culture medium for the entirety of cell culture (see Chapter 2). In contrast to CD25 expression, CD107a did not continue to increase after 4 hours and remains at a comparable level through at least 18 hours of culture (Figure 7B). This indicates that we can also measure CD107a upregulation after 18 hours, despite it also not being a true peak of expression. This is consistent with the mechanics of including anti-CD107a in the culture medium; CD107a is captured by the anti-CD107a at the cell surface when trafficked to the plasma membrane during degranulation. Thus the CD107a read-out represents the total percentage of CD107a-expressing cells during culture up until the time of cell harvest [20], rather than a cross-sectional snap-shot as with CD25.

It should be acknowledged that HCC is not the optimal positive control for measuring degranulation responses, which are better induced by direct contact through ligation of activating receptors rather than exogenous cytokines. It is therefore possible that the kinetics of the degranulation response may be different with another route of activation, e.g.

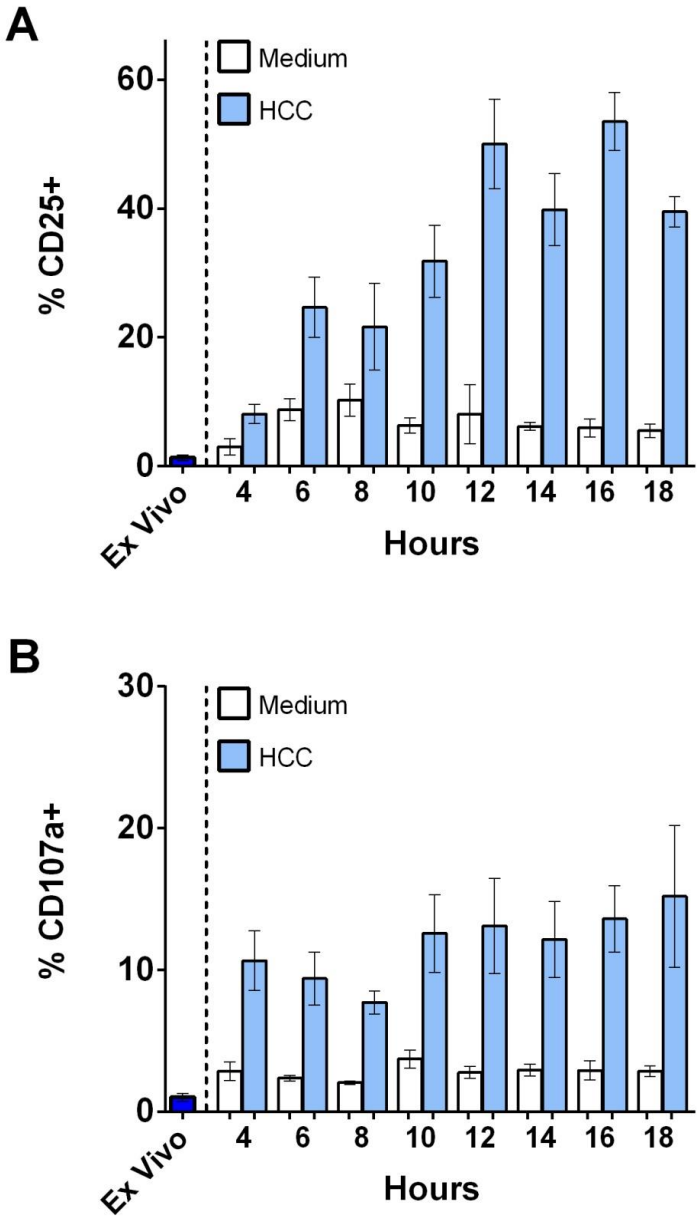

Figure 7. Time series of CD25 and CD107a expression by NK cells following stimulation with high concentrations of IL-12 and IL-18. PBMC were cultured for 18 hours in medium alone or in the presence of a high concentration of cytokines (HCC: 5ng/ml IL-12, 50ng/ml IL-18). Bars represent means with error bars corresponding to SEM (standard error of mean). *n* = 5. Data are from a single experiment.

crosslinking of NK cell activating receptors, such as CD16. Evaluation of NK cell responses to CD16 crosslinking after 6 hours and 18 hours is included in Chapter 5.

3.3.4 Selecting vaccine antigens

To investigate the capacity of heterogeneous NK cells to contribute to secondary responses to vaccine antigens, it was necessary to select specific vaccines to use as models in our *in vitro* assays. Previous work in our group had used tetanus toxoid, which had induced low NK cell responses (data not shown). As a subunit vaccine, we postulated that the poor activation in culture may be related to the relative lack of PAMPs and consequent paucity of accessory cell stimulation. We therefore decided to conduct pilot experiments with a wide range of vaccines, including viral and bacterial whole pathogen vaccines, as summarised in Table II.

Cryopreserved PBMC from three donors were thawed, rested, and stimulated for 18 hours with titrations of each of the ten vaccines, with or without LCC. Upregulation and co-expression of surface CD25 and intracellular IFN-γ was used to detect functional NK cell activation, as discussed above, and HCC was used as a positive control.

Two main observations were made from this pilot experiment. Firstly, the majority of the live vaccines (measles, mumps, rubella) appeared to negatively affect the lymphocyte population — particularly the NK cells — during culture in a dose-dependent manner, as illustrated by the FACS plots in Figure 8. Although we cannot be certain this is due to cell death in the absence of a live/dead marker in our staining panel, this seems a reasonable conclusion given the same trend is observed with pertussis toxin. It is also possible that a contaminant from the vaccine preparation is adversely affecting the PBMC population rather than the viruses themselves, and further investigation of this hypothesis would be warranted if studies with these specific vaccines were pursued in the future. Of the remaining vaccines, responses were higher to the whole pathogen formulations (Figure 9). Specifically, NK cell activation was most robust in

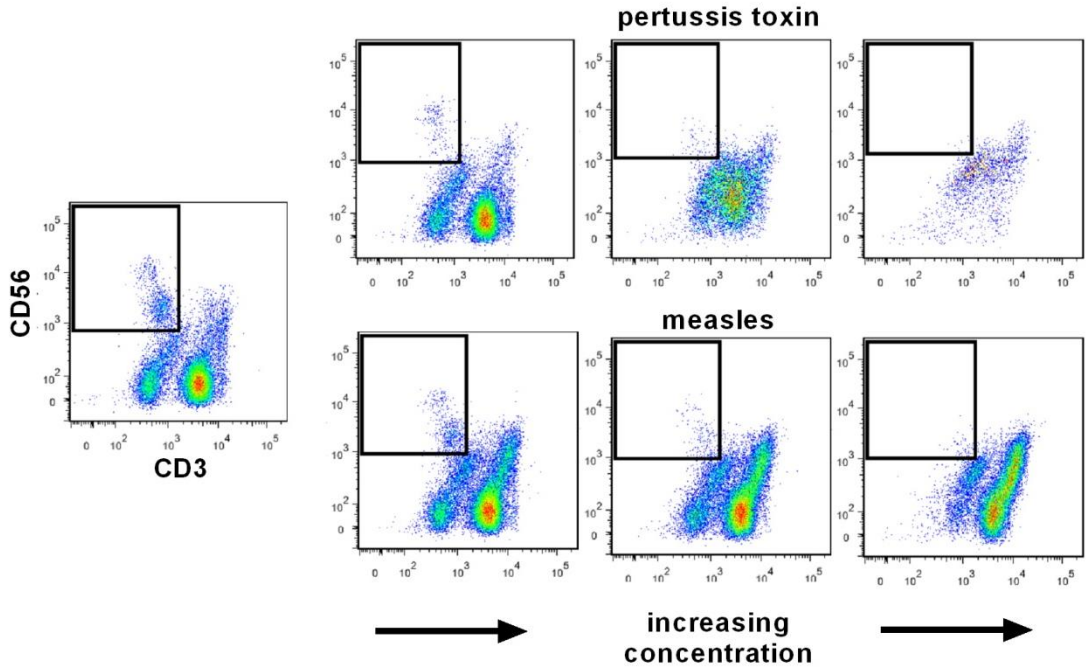

Figure 8. Culture with live measles vaccine or pertussis toxin (PT) adversely affects PBMC population. Flow cytometry plots showing gating of NK cells from total lymphocyte populations after 18 hours culture with increasing concentrations of live measles virus vaccine (400IU/ml, 800IU/ml, 1600IU/ml) or pertussis toxin (2ng/ml, 20ng/ml, 40mg/ml) for one donor. Similar trends were observed with other donors and live mumps or rubella vaccines.

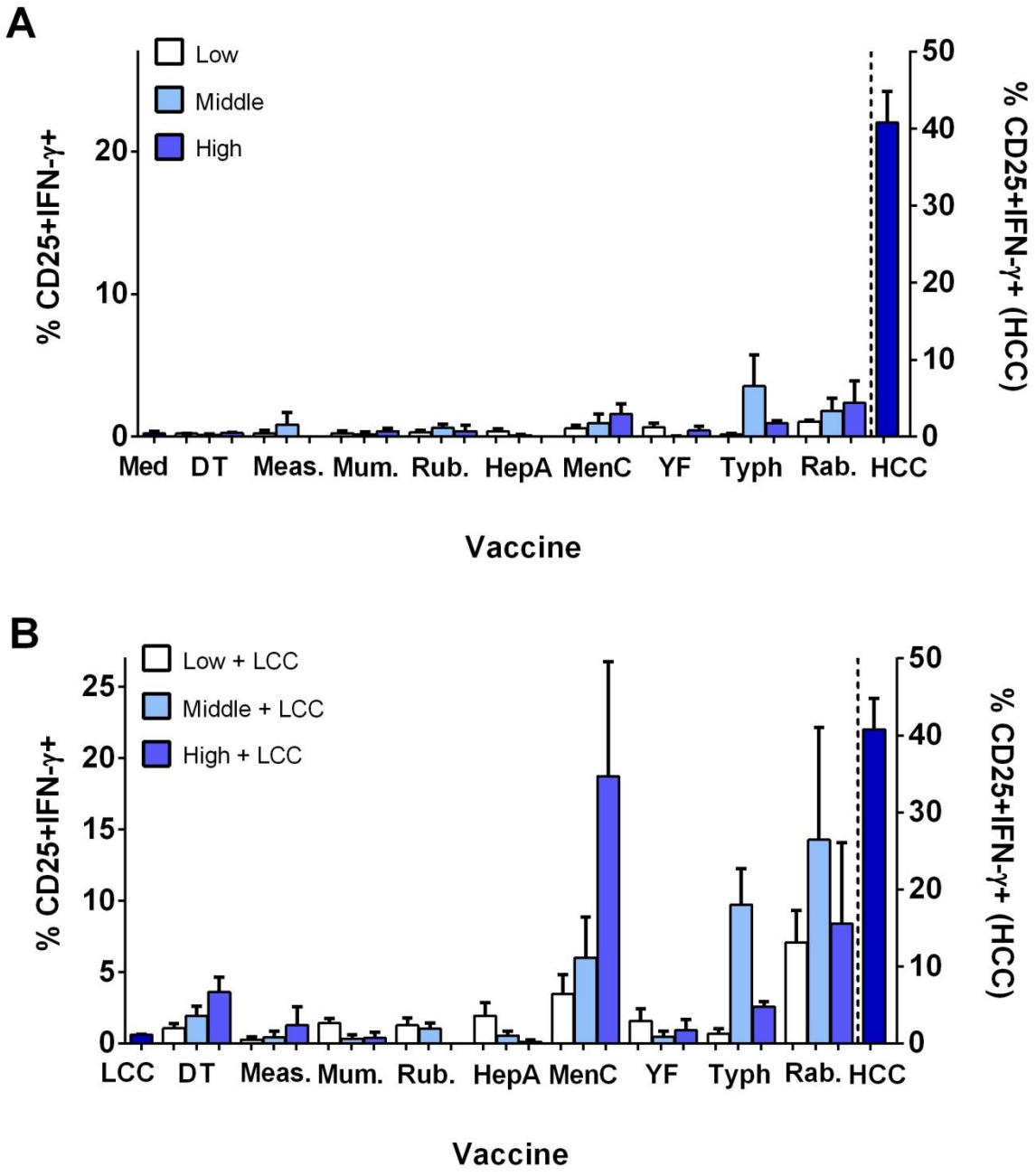

Figure 9. CD56dimCD57- NK cell responses to titration of vaccines. PBMC were cultured for 18 hours in **(A)** medium alone, and increasing concentrations (low, medium, high; see Table II) of DT (diphtheria toxoid), meas. (lives measles virus), mum. (live mumps virus), rub. (live rubella virus), HepA (inactivated whole virus hepatitis A), MenC (meningococcal C polysaccharide), YF (live yellow fever virus), typh (killed whole cell typhoid), rab. (killed whole virus rabies), or **(B)** with a low concentration of cytokines (LCC: 12.5pg/ml IL-12 and 10ng/ml IL-18) and these same vaccine antigens. A high concentration of cytokines (HCC; 5ng/ml IL-12 and 50ng/ml IL-18) was used as positive control. CD56dimCD57- NK cells were analysed by flow cytometry for co-expression of CD25/IFN-γ. Bars represent means with error bars corresponding to SEM (standard error of mean). *n* = 3. Data are from a single experiment but similar titrations were performed on other occasions.

response to inactivated whole virus rabies, inactivated whole H1N1 influenza virus (not shown), and killed whole cell typhoid. Subsequent experiments with killed whole cell pertussis, which contains inactivated pertussis toxoid rather than the toxin, were consistent with this. As ongoing work in our group was already optimising working concentrations of H1N1 influenza (ultimately selected to be 1μg/ml) and to be consistent with previous experiments with tetanus toxoid (7.5μg/ml), further titration work was only performed with whole cell pertussis and diphtheria toxoid (Figure 10). Despite low responses in early pilot work, diphtheria toxoid was included to allow comparison of all components of the ubiquitous childhood vaccine DTP (diphtheria-tetanus-pertussis). Although responses to meningococcal C polysaccharide were among the most robust, this was not included for further experiments to limit the size of the assays. NK cells were thus analysed and responses were detectable to both diphtheria toxoid and killed whole cell pertussis. The higher concentrations (diphtheria toxoid- 1μg/ml; whole cell pertussis- 1IU/ml) gave marginally higher responses and were thus selected for further work.

Section B

The following work is adapted from White*, Nielsen* *et al* ([1], Appendix II), with the exception of section *3.3.9 Autologous plasma enhances NK cell responses to vaccine antigens* (unpublished data).

3.3.5 DTP vaccination induces durable vaccine antigen-driven NK cell responses

To validate DTP vaccination as a suitable model for evaluating NK cell recall responses, PBMC were incubated overnight with tetanus toxoid, diphtheria toxoid, or killed whole cell pertussis with or without low concentrations of the cytokines IL-12 and IL-18 (LCC) or, as a positive control, with a high concentration of cytokines IL-12 and IL-18 (HCC), stained for NK cell phenotypic and functional markers, and examined by flow cytometry (Figure 11). HCC induces

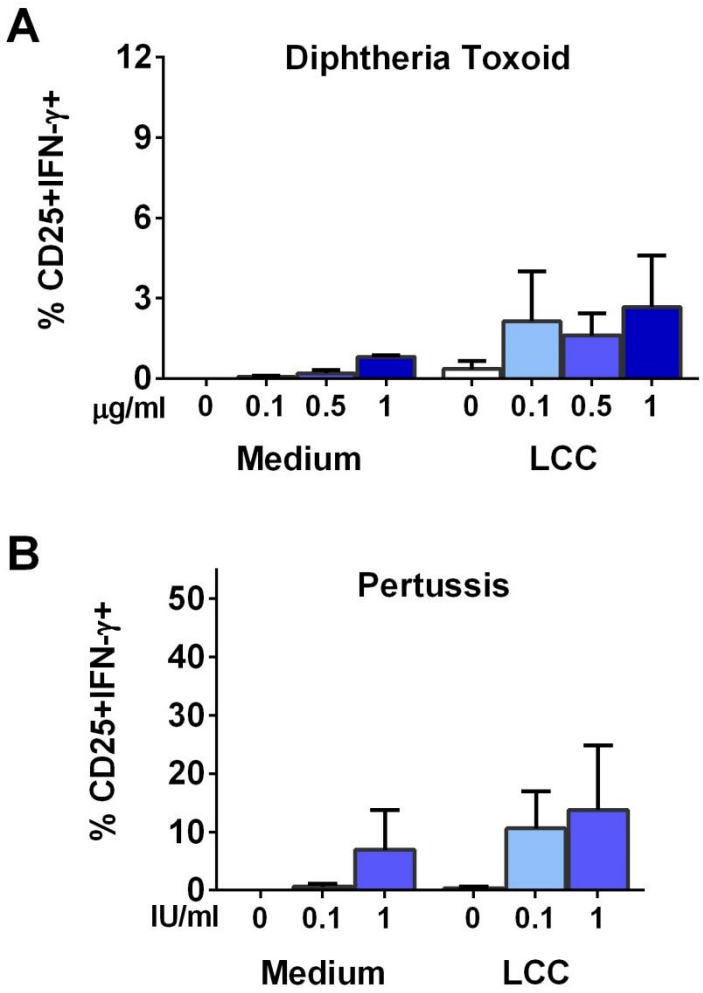

Figure 10. NK cell responses to diphtheria toxoid or killed whole cell pertussis. PBMC were incubated for 18 hours in medium alone (Med), low concentration of cytokines (LCC: 12.5pg/ml IL-12 and 10ng/ml IL-18), 0.1, 0.5 or 1µg/ml diphtheria toxoid with or without LCC **(A)**, or 0.1 or 1IU/ml killed whole cell pertussis with or without LCC **(B)**. NK cell responses were measured as the percentage of cells co-expressing CD25/IFN-γ. Bars represent means with errors bars corresponding to SEM (standard error of mean). *n* = 3. Data are from a single experiment but similar titrations were performed on other occasions.

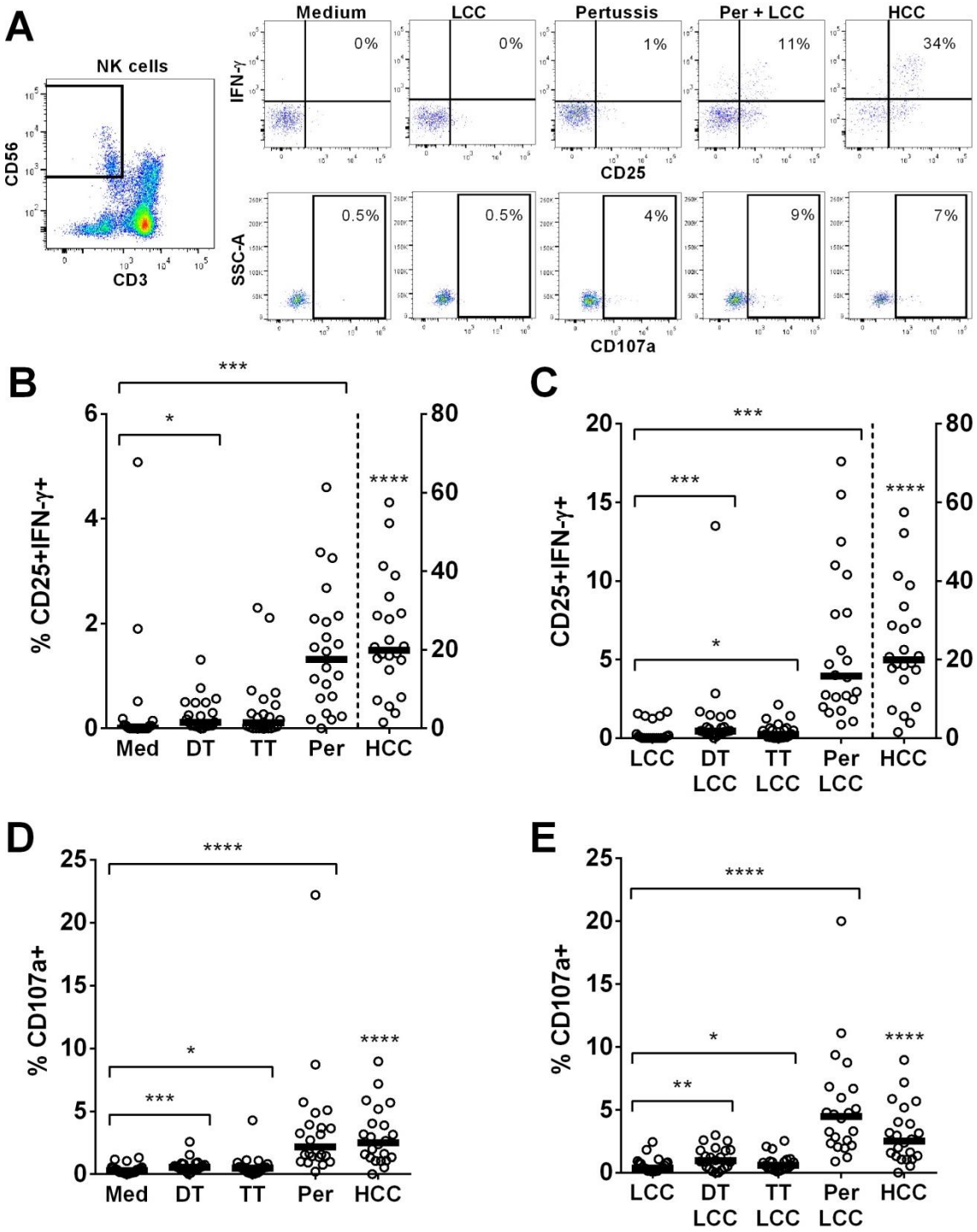

Figure 11. NK cell responses to diphtheria toxoid, tetanus toxoid and killed whole cell pertussis. PBMC were cultured for 18 hours in medium alone (Med), low concentrations of IL-12 and IL-18 (LCC; 12.5pg/ml IL-12, 10ng/ml IL-18), tetanus toxoid (TT), diphtheria toxoid (DT), killed whole cell pertussis (Per), TT and LCC, DT and LCC, Per and LCC, or high concentrations of IL-12 and IL-18 (HCC; 5ng/ml IL-12, 50ng/ml IL-18). Representative flow cytometry plots show gating of CD3+CD56+ NK cells, co-expression of CD25 and IFN-γ, and upregulation of CD107a **(A)**. NK cell responses were measured following 18 hours culture as percentage of cells co-expressing CD25/IFN-γ following stimulation with vaccine alone **(B)**, vaccine with LCC **(C)**, or upregulation of CD107a to vaccine alone **(D)**, or vaccine with LCC **(E)**. Note that in **(B)** and **(C)** the HCC responses are shown on the right-hand axis. Each data point represents one donor, *n* = 22. Lines represent median values. Data were analysed with paired, one-tailed, non-parametric Wilcoxon signed rank tests. **** $p \leq 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

up to 60% of CD3-CD56+ NK cells to express cell surface CD25 and intracellular IFN-γ (Figure 11A-C) and has a significant, but much less marked, effect on CD107a expression (Figure 11A,D-E). By contrast, LCC alone induces a small, though significant, proportion of NK cells to express CD25, but few, if any, of these cells also produce IFN-γ or express CD107a on their surface (Figure 11A). This is consistent with use of LCC as a booster for antigen-driven IFN-γ or CD107a NK cell responses.

Among PBMC stimulated with vaccine antigen alone (i.e. without LCC) there is highly significant upregulation of both CD25 and IFN-γ by NK cells in response to pertussis, a lesser (but still significant) response to diphtheria toxoid and no significant response to tetanus toxoid (Figure 11B). However, responses to all three antigens were significantly enhanced in the presence of LCC (Figure 11C). These data are fully consistent with a scenario in which a whole cell antigen such as pertussis contains ligands for toll-like receptors (TLRs) [21] and thus induces accessory cells to secrete cytokines such as IL-12 and IL-18, whereas purified proteins such as tetanus and diphtheria toxoids do not; exogenous LCC induces expression of CD25 (and thus the high affinity IL-2R) on NK cells allowing them to respond to IL-2 from vaccine-specific CD4+ T cells. By contrast, a statistically significant increase in CD107a expression on NK cells was seen response to all three vaccine components (Figure 11D) and this was not sunstantially enhanced by LCC (Figure 11E).

3.3.6 CD56 and CD57 define multiple distinct NK cell subsets

Despite very robust NK cell responses to some of the DTP vaccine antigens, not all NK cells responded and there is considerable heterogeneity in the magnitude of the NK cell response between donors (Figure 11B-E). Whilst heterogeneity between individuals might be explained by variation in the strength of the T cell IL-2 response that drives the NK responses [4,22,23] this is unlikely to explain heterogeneity of responses within the NK cell population of an individual donor. We therefore considered whether within-donor variation might be due to

differences between subsets of NK cells in their intrinsic sensitivity to activation by monokines and T cell derived IL-2.

CD57 is a marker of highly differentiated, highly cytotoxic NK cells [14,24,25]. Expression of CD56 and CD57 has been used to identify three subsets of NK cells. Functional analysis of these subsets suggests that NK cells differentiate from relatively immature CD56brightCD57- cells which respond to cytokine stimulation by producing IFN-γ but have limited cytotoxic potential, to CD56dimCD57- cells which are also poorly cytotoxic but retain IL-2Rα (CD25) expression and thus the ability the secrete IFN-γ in response to cytokine stimulation and, eventually, to CD56dimCD57+ cells which no longer respond to exogenous cytokines but are skewed towards a cytotoxic phenotype following crosslinking of CD16 or other activatory receptors or exposure to target cells [12,14,24]. However, CD57 expression is not simply 'off' or 'on' but is gradually upregulated in a stepwise fashion (Figure 12 [1]). While we have demonstrated the stability of CD57 in an 18 hour culture [1], the stimuli involved in upregulating CD57 over the longterm and thus involved in driving this stepwise maturation remain of interest. This is the focus of a separate paper by our group (White *et al*, manuscript in preparation).

Most importantly, the functional remodelling of NK cells in terms of loss of cytokine-induced upregulation of CD25 and IFN-γ expression, is extremely gradual with complete unresponsiveness to HCC not being seen until CD57 expression reaches its maximal level [1]. By contrast, little or no difference was observed in the ability of NK cells with different levels of CD57 expression to degranulate in the presence of cytokines [1]. This is in contrast to data from more traditional degranulation assays that stimulate NK cells by crosslinking activatory receptors [14], where acquisition of CD57 is associated with increased – not merely maintained – cytotoxic potential. This data is not inconsistent, but rather points to a significant role for cytokine co-stimulation, and thus cytokine sensitivity, in degranulation response to vaccine antigens.

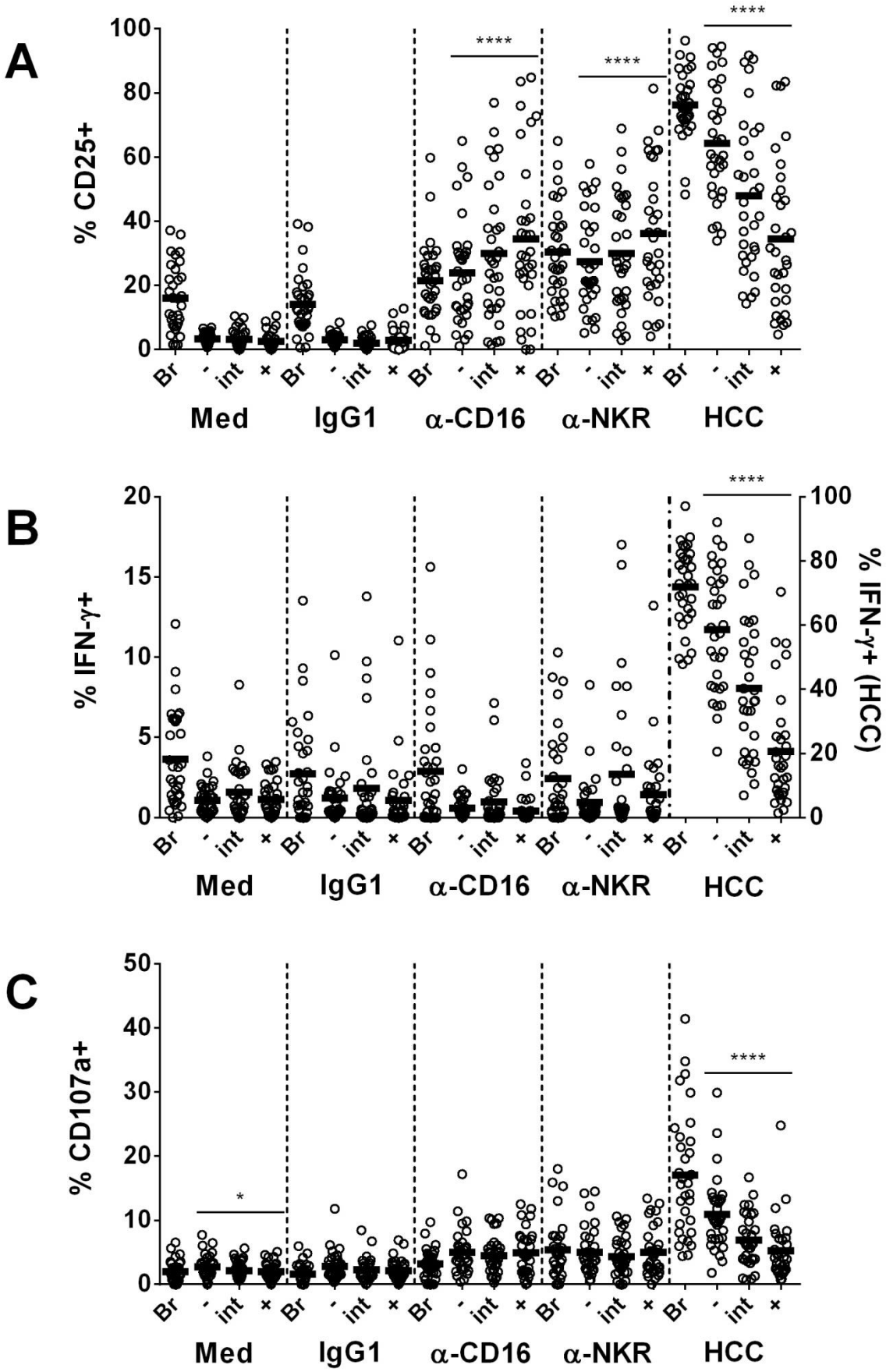

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Figure 12. CD57 defines a continuum of functionally distinct NK cells. PBMC were cultured for 18 hours in medium alone, or with crosslinking antibodies to monoclonal IgG1 (isotype control), CD16, a combination of NK cell receptors (NKR: NKG2D, NKp30, NKp46, 2B4), or high concentrations of IL-12 and IL-18 (HCC; 5ng/ml IL-12, 50ng/ml IL-18). Representative flow cytometry plots show gating of CD56bright (Br) and CD56dim NK cells, and subsequent gating of the CD56dim subset into CD56dimCD57- (-), CD56dimCD57intermediate (int) and CD56dimCD57+ (+) populations are shown in Figure 2. NK cell responses were measured as the percentage of cells expressing CD25 **(A)**, IFN-γ **(B)**, or CD107a **(C)**. Note that in **(B)** the HCC responses are shown on the right-hand axis. Each data point represents one donor, n = 33. Lines represent mean values. CD56dim subsets were analysed for linear trend with a repeated measures ANOVA. **** $p \leq 0.0001$, * $p < 0.05$.

Our data also suggest that NK cells with intermediate levels of CD57 expression (CD57int), which represent a significant fraction (~30%) of circulating NK cells, are also intermediate in terms of their functional maturation. To formally test this hypothesis, we analysed responses of the four NK cell subsets (CD56bright; CD56dimCD57-; CD56dimCD57int and CD56dimCD57+, Figure 12) to HCC, crosslinking of CD16 and crosslinking of NK receptors, by expression of CD25, IFN-γ, or CD107a (Figure 12). As expected, high proportions of CD56bright cells expressed CD25, IFN-γ or CD107a in response to HCC; crosslinking of CD16 or other activatory receptors upregulated CD25 and CD107a but not IFN-γ in this subset (Figure 12). Among CD56dim NK cells, CD25, CD107a and IFN-γ responses to HCC declined with increasing levels of CD57 expression with a statistically significant negative trend from CD56dimCD57- cells, through CD56dimCD57int cells to CD56dimCD57+ cells (ANOVA for all linear trends, $p \leq 0.0001$; Figure 12).

Interestingly, although no significant differences were observed between the three CD56dim populations in their ability to degranulate or produce IFN-γ in response to CD16 or NKR crosslinking, crosslinking of CD16 or NKRs led to increasing levels of CD25 expression with increasing expression of CD57 (linear trend; $p \leq 0.0001$ in both cases), suggesting that responsiveness to T cell IL-2 may be retained in CD56dimCD57+ NK cells in the presence of antibodies able to induce ADCC. In summary therefore, the transition from CD56bright to

CD56dim correlates with an immediate switch from cytokine secretion to cytotoxicity in response to crosslinking of NKRs or CD16 receptors whereas increasing CD57 expression correlates with a gradual loss of responsiveness to IL-12 and IL-18.

3.3.7 Vaccine-driven, cytokine-mediated NK IFN-γ responses are dominated by the CD56dimCD57- and CD56dimCD57int NK cell subsets

Accessory cytokines (including IL-12 and IL-18) and T-cell-derived IL-2 are known to be essential to drive NK cell IFN-γ responses during re-stimulation with vaccine antigens [4]. Given that increasing CD57 expression correlates with loss of responsiveness to HCC, we predicted that CD56dimCD57- or CD56dimCD57int NK cell populations would show stronger 'recall' responses to whole cell pertussis than would CD56dimCD57+ NK cells. To test this hypothesis, responses to pertussis (Figure 11) were analysed for each of the four NK cell subsets defined by CD56 and CD57 expression (Figure 13). There was a clear hierarchy of responses with a significantly higher proportion of CD56dimCD57- NK cells than CD56dimCD57int or CD56dimCD57+ NK cells co-expressing CD25 and IFN-γ ($p < 0.001$ for linear trends; Figure 13A). On the other hand, CD107a expression was similar among all three CD57-defined NK cell subsets (Figure 13C,D). When considering the proportion of all NK cells belonging to each subset together with the responsiveness of each individual subset, it becomes evident that vaccine antigen-driven NK cell IFN-γ recall responses occur almost entirely within the CD56bright and CD56dimCD57- NK cell subsets with minimal contribution from the CD56dimCD57int and CD56dimCD57+ subsets (Figure 13B).

It is therefore clear that interdonor variation in NK cell vaccine responsiveness will be greatly impacted by the distribution of CD56/CD57-defined subsets in individuals. We therefore anticipate that all factors that influence this distribution (as I review in [8]), such as ageing [7,26-32] or HCMV infection [33-36], will affect NK cell vaccine responsiveness at the level of the total NK cell population. This hypothesis is explored further for cytomegalovirus in the

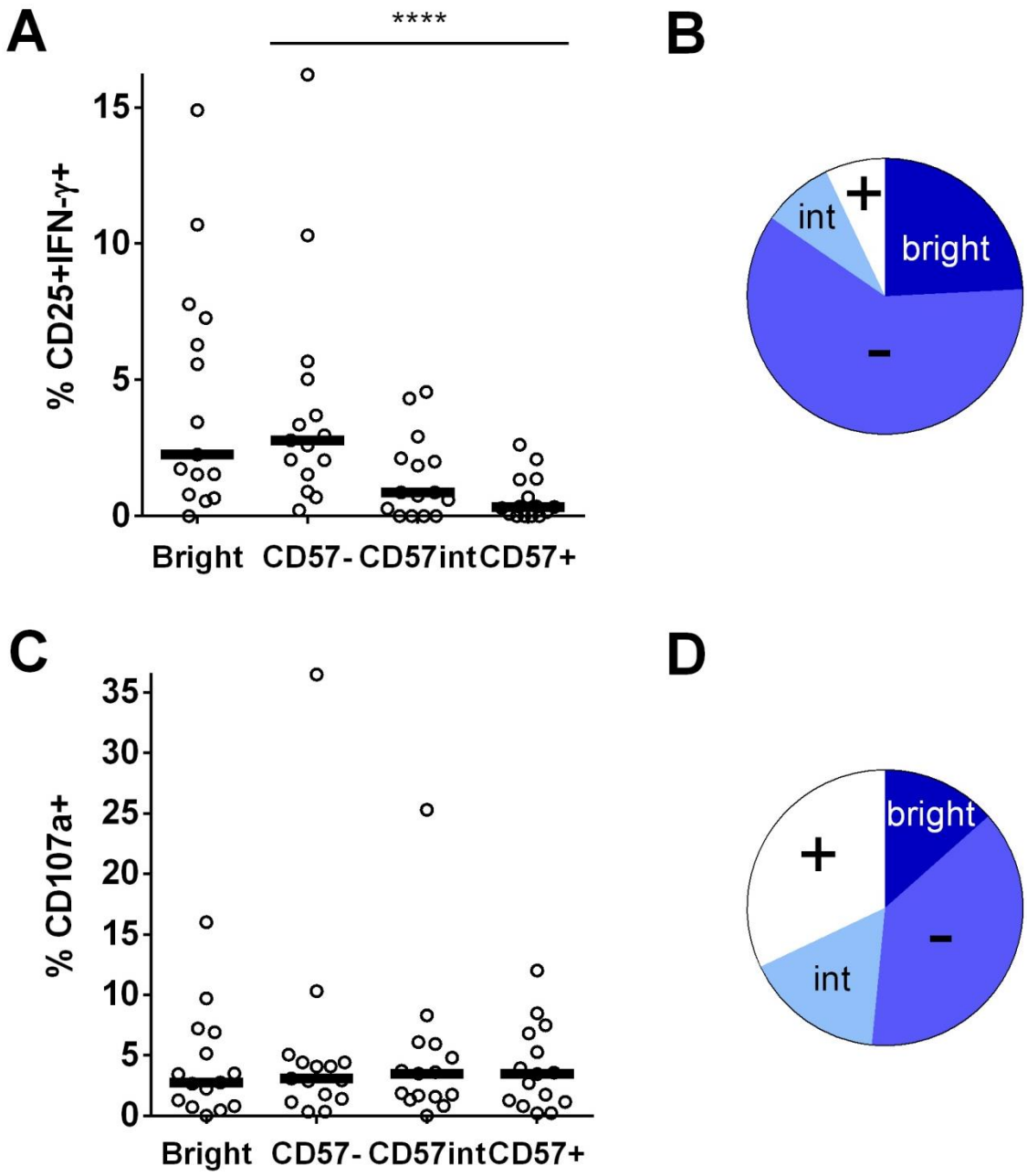

Figure 13. NK cell IFN-γ responses to pertussis are dominated by the CD56dimCD57- NK cell subset. PBMC were cultured with killed whole cell pertussis for 18 hours. The percentage of cells in CD56/CD57-defined subsets responding are defined as CD25+IFN-γ+ **(A-B)** or CD107a+ **(C-D)**. Pie charts illustrate the mean percentage of the total responding NK cells attributable to each subset for CD25+IFN-γ+ **(B)** and CD107a+ **(D).** Each data point represents one donor, *n* = 15. Lines represent median values. CD56dim subsets were analysed for linear trend with a repeated measures ANOVA. ****$p < 0.001$.

following chapter. Work by our group on the contribution of ageing to NK cell heterogeneity has also been published and is discussed further in Chapter 4, and included as Appendix VIII to this thesis [37].

3.3.8 NK cell acquisition is associated with reduced expression of cytokine receptors IL-12Rβ2 and IL-18Rα

CD57 acquisition on NK cells is associated with a reduced ability to respond to accessory cytokines (Figure 12) leading to a progressive decline in their ability to respond to vaccine-driven cellular responses by production of IFN-γ (Figure 13A). To determine whether this is due to altered cytokine receptor expression and/or altered downstream signalling we assessed the resting (*ex vivo)* expression of IL-18Rα and IL-12Rβ2 (Figures 14). These receptor components were selected as the specific binding subunit or inducible signalling subunit of the IL-18R and IL-12R respectively (further discussion of cytokine receptor subunit kinetics is presented in Chapter 5). In the absence of a commercially available fluorescently-labelled IL-12Rβ2 antibody, the anti-IL-12Rβ2-antibody was PerCP-Cy5.5 conjugated in house. The isotype control staining and titrations for this antibody and were performed by Martin Goodier (Appendix IX).

The proportion of IL-12Rβ2 expressing cells was highest among the CD56bright NK cells with a progressive decrease in expression across the CD57 defined NK cell subsets (Figure 14B) but IL-12Rβ2 expression density did not vary across subsets (Figure 14C). Although IL-18Rα was expressed at a much higher frequency than IL-12Rβ2 within all NK cell subsets, the same trend was seen, with declining IL-18Rα expression with increasing CD57 expression (Figure 14D). In contrast to IL-12Rβ2, however, IL-18Rα mean fluorescence intensity also declined with increasing CD57 expression (Figure 14E). This suggests fewer cells in CD57+ subset express IL18Rα and — of those that do — the expression of IL18Rα is lower, whereas while fewer cells

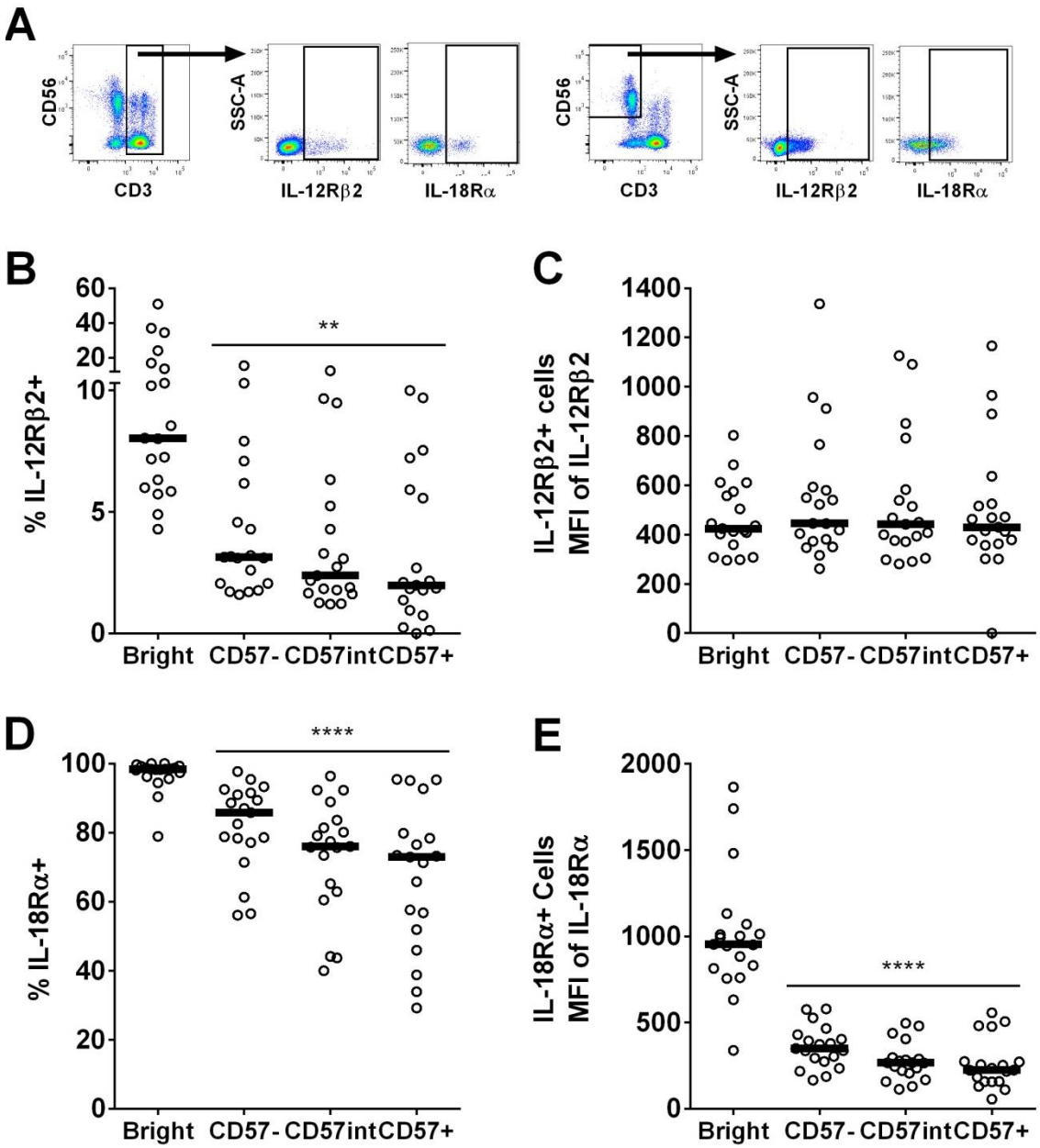

Figure 14. IL-12Rβ2 and IL-18Rα expression decrease with CD57 expression. PBMC were analysed *ex vivo* for IL-12Rβ2 and IL-18Rα expression. Representative flow cytometry plots show gating for IL-12Rβ2 and IL-18Rα: gates were set from CD3+ (T cell) population (left) and copied to NK cell populations (right) **(A)**. Frequency **(B)** and mean fluorescence intensity (MFI) **(C)** of IL-12Rβ2expression, and frequency **(D)** and MFI **(E)** of IL-18Rα expression, were assessed by subset. Each data point represents one donor, n = 19. Lines indicate median values. CD56dim subsets were analysed for linear trend with a repeated measures ANOVA. **** $p \leq 0.0001$, ** $p < 0.01$. The *in vitro* cell culture and staining for this experiment were performed by Matt White and Scarlett Turner.

in CD57+ subset express IL-12Rβ2, the expression on a per cell basis is similar to on the less mature subsets.

3.3.9 Autologous plasma enhances NK cell responses to vaccine antigens

The data reported above all come from cell culture experiments where the RPMI medium was supplemented with 10% pooled human AB plasma. This is a standard cell culture system, although some groups use foetal bovine/ calf serum (FBS/ FCS) to support cell culture instead. The use of AB plasma means there is a consistent level of antibody, and other plasma components, across all of the assays. Antibody levels, particularly IgG, are clearly of importance in the context of evaluating ADCC responses; indeed no ADCC would be possible in FCS-supplemented plasma, due to the lack of IgG to form antigen-antibody complexes with which to crosslink CD16. However, the use of pooled plasma does obscure true differences in responses between donors in the context of their own plasma. For example, if a subject has particularly high titres of anti-pertussis IgG, then we would anticipate more robust ADCC responses if cells are cultured in autologous plasma as compared to low-titre plasma or pooled AB. We therefore simultaneously performed the vaccine antigen stimulation experiments in autologous plasma, alongside the assays in AB plasma, to evaluate the effect of autologous plasma on NK cell responses and whether there was correlation with antibody titres.

PBMC from a total of 52 healthy adults (i.e. 19 additional donors to the experiments presented above) were stimulated for 18 hours with killed whole cell pertussis with or without LCC, in either 10% pooled AB plasma or 10% autologous plasma. IgG titres to pertussis toxin (PT) were measured in AB plasma (6IU/ml) and autologous plasma for each donor (range 1.4-81.4IU/ml, mean = 11.4IU/ml); see Chapter 4 for detailed ELISA methods. NK cell responses in terms of CD107a and CD25+IFN-γ+ upregulation were assessed using flow cytometry (Figure 15). It is immediately obvious that use of autologous instead of AB plasma greatly boosts both CD107a and CD25+IFN-γ+ NK cell responses; for example, the mean CD107a responses to pertussis in

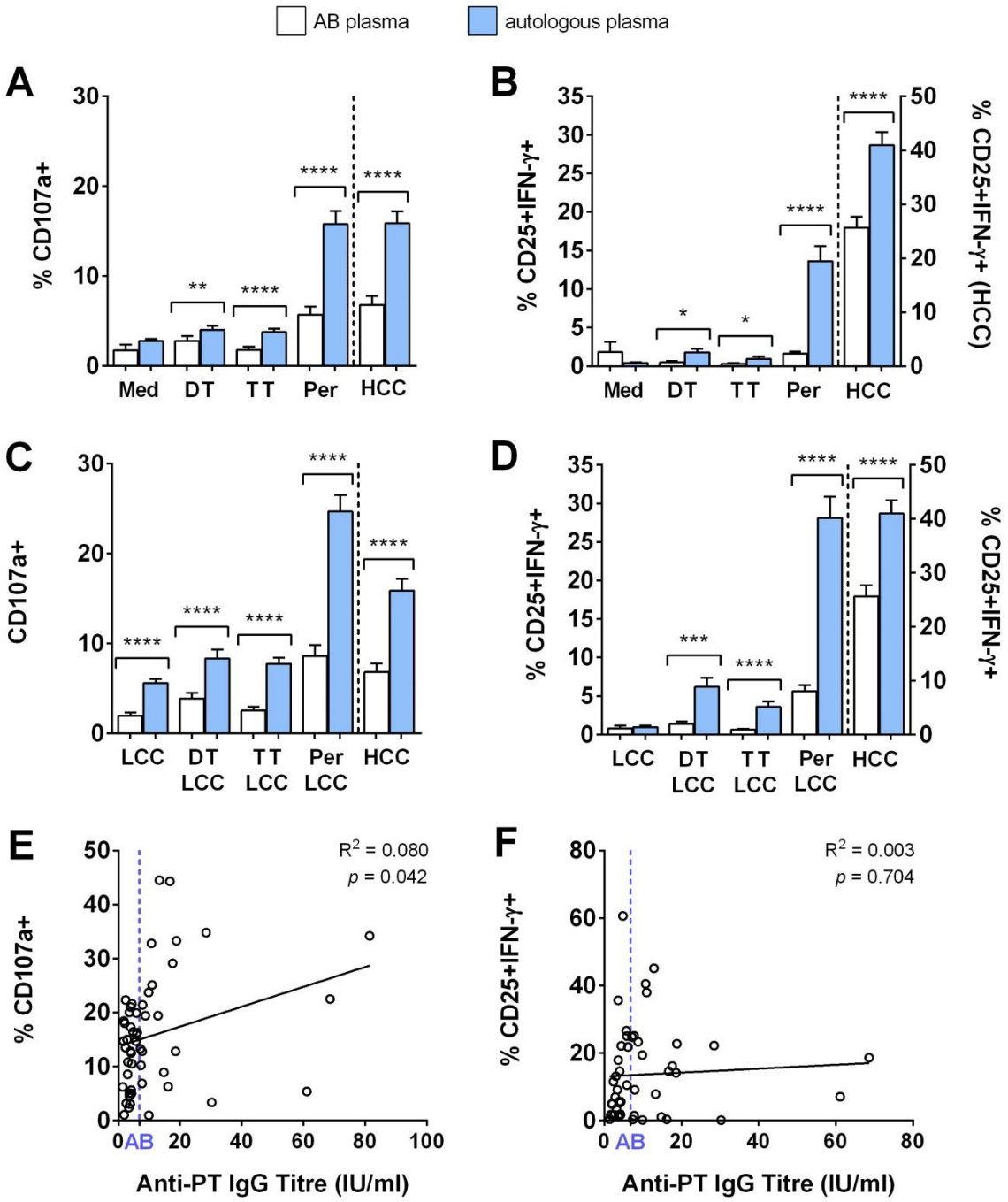

Figure 15. Correlation between NK cell responses to pertussis in autologous plasma and anti-PT IgG titre. PBMC were cultured for 18 hours in medium alone, low concentrations of IL-12 and IL-18 (LCC; 12.5pg/ml IL-12, 10ng/ml IL-18), tetanus toxoid (TT), diphtheria toxoid (DT), killed whole cell pertussis (Per), TT and LCC, DT and LCC, Per and LCC, or high concentrations of IL-12 and IL-18 (HCC; 5ng/ml IL-12, 50ng/ml IL-18), in pooled human AB or autologous (Auto.) plasma. IgG titres to pertussis toxin (PT) were measured in AB and autologous plasma using an in-house ELISA (see Methods in Chapter 4). The percentage of NK cells expressing CD107a **(A,C,E)** or co-expressing CD25/IFN-γ **(B,D,F)** were compared between cultures in AB and autologous plasma following stimulation with vaccine alone **(A,B)** or vaccine with LCC **(C,D)**. Anti-PT IgG titres in autologous plasma were correlated against the percentage of NK cells expressing CD107a **(E)** or co-expressing CD25/IFN-γ **(F)** after culture with pertussis alone in autologous plasma. The anti-PT IgG titre of AB plasma is annotated for reference **(E, F)**… **(Figure legend continued page 85).**

(continued from page 84)
Each point represents a single donor, n = 52. Bars represent means with errors bars corresponding to SEM (standard error of mean). NK cell responses in AB and autologous plasma were compared using paired t-tests. Correlation of NK cell responses with anti-PT IgG titres was assessed using linear regression; R^2 and p values are as annotated on graphs. **** $p \leq 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

AB plasma were 5.7% and 15.8% in autologous plasma ($p < 0.0001$, paired t test, Figure 15A), while the mean CD25+IFN-γ responses were 1.6% and 13.6% respectively ($p < 0.0001$, paired t test, Figure 15D).

While there is weak correlation between CD107a responses and anti-PT titres in autologous plasma ($R^2 = 0.080$, $p = 0.042$; Figure 15C), there is no correlation between CD25+IFN-γ+ upregulation and anti-PT IgG titre ($R^2 = 0.003$, $p = 0.704$; Figure 15F). This is consistent with a greater role for IgG in driving ADCC or degranulation, as measured by CD107a, than IFN-γ production (see Chapter 4 for further discussion of this dichotomy), but the weak correlation between the CD107a response and anti-PT titres also implies that little antibody is required to drive optimal degranulation. In the absence of strong associations between IgG titres and NK cell functionality, these data suggest there is a difference between autologous and AB plasma other than higher anti-PT antibody levels that is driving the enhanced NK cell responses to killed whole cell pertussis in autologous plasma. Indeed, the anti-PT titre of AB plasma is 6IU/ml which is a mid-range titre as compared to the autologous plasma titres (as would be expected from pooled plasma), but results in NK cell responses significantly lower than in autologous plasma for all donors.

This capacity of autologous plasma to boost NK cell responses or, perhaps more accurately, the dampening of NK cell responses in AB plasma is intriguing. Unfortunately, a thorough analysis of the components of AB plasma preparations and the differences with autologous plasma was not feasible at the time, but would make an interesting future project. In the absence of a clear

mechanism by which autologous plasma improves NK cell responses in autologous plasma, it was decided to proceed using only AB plasma-supplemented media to standardise assays.

3.4. Discussion

Vaccination typically provides long-lasting protection against infectious diseases by inducing the expansion and differentiation of small populations of naïve, antigen-specific, T and B cells into much larger populations of long-lived memory cells with enhanced effector function. In particular, antigen-specific memory CD4+ T cells augment B cell, CD8+ T cell and macrophage-mediated effector functions [38]. Although circulating antibody may persist for many years after vaccination, frequencies of antigen-specific memory T cells are typically extremely low in peripheral blood (approximately 1 in 10,000 [39]) and can be difficult to detect in the absence of recent boosting. However, the observation that IL-2 produced in an antigen-specific manner by CD4+ T cells can activate a substantial proportion (varying from ≈1% up to 60% in some cases) of all circulating NK cells [3,4,15,22,40], and that these responses can be detected for more than 20 years after vaccination in the case of DTP, suggests that NK cell responsiveness might represent a more sensitive biomarker of T cell induction and maintenance and might thus have a role to play in evaluation of new vaccines or new vaccine formulations. Whether NK cells – activated by T cell IL-2 or by crosslinking of Fc Receptors (CD16) by immune complexes – play an important role as effectors of vaccine induced immunity is as yet unknown but the speed with which they are activated (within six hours of exposure to the pathogen [4]) and the large number of potentially responding cells suggests that their role should be investigated.

We observed that NK cell responses to pertussis were significantly greater in magnitude than responses to diphtheria toxoid or tetanus toxoid, even though all three antigens would have been administered together during vaccination. A likely explanation for this is that the pertussis antigen is a whole cell preparation containing numerous ligands for pattern recognition receptors on macrophages and dendritic cells, leading to their secretion of IL-12 and IL-18, which is necessary to induce NK cells to secrete IFN-γ and become cytotoxic [5,15]. Purified toxoids such as diphtheria toxoid and tetanus toxoid lack such ligands and thus, *in*

vitro at least, NK cells can only be induced to respond in the presence of exogenous IL-12 and IL-18. *In vivo* however, infection by live tetanus and diphtheria bacteria would presumably induce a strong accessory cell cytokine response. On the other hand, much stronger NK responses to pertussis than diphtheria toxoid or tetanus toxoid were seen even in the presence of low concentrations of IL-12 and IL-18, suggesting that whole cell pertussis may also induce a stronger T cell response than does a toxoid antigen.

Despite an overall tendency for NK cells to respond to vaccine antigens, there was considerable heterogeneity between individuals which may in part be explained by inter-individual variation in T cell IL-2 responses. However, we also observed heterogeneity between NK cell subsets in their responsiveness to vaccine-driven signals, with responses being dominated by CD56bright and CD56dimCD57- NK cells. This correlated with higher levels of CD25 expression on IL-12/IL-18 activated CD57- cells compared to CD57+ cells and a higher resting level expression of IL-12Rβ2 and IL-18Rα on these cells. These findings are in line with previous reports that CD57+ NK cells are less able to respond to cytokines [12,14], and express lower levels of IL-18Rα and lower amounts of mRNA for IL-12Rβ2, compared to CD57- NK cells. The established model is that IL-18 induces expression of the high affinity IL-2Rα (CD25) on NK cells [41] whilst IL-12 is necessary, but not sufficient for their production of IFN-γ [42]. Moreover, IL-2 induces expression of the inducible chain of the IL-12R (IL-12β2) [43]. Thus, as shown here, synergy between these three cytokine signals, IL-2, IL-12 and IL-18, results in NK cells producing high levels of IFN-γ during the first 18-24 hours following re-exposure to vaccine antigens. Further work on the relationship between IL-2, IL-12 and IL-18 in the context of NK cell activation by vaccine antigens is presented in Chapter 5.

Of interest, we have observed that the maturation of NK cells from CD56brightCD57- to CD56dimCD57+ is a gradual process with functional changes being highly correlated with CD56 and CD57 expression. This is particularly apparent for the cytokine driven pathway of NK cell

activation where expression of IL-12R and IL-18R as well as IL-12/IL-18-induced CD25 expression and IFN-γ synthesis are all very tightly negatively associated with CD57 expression. We find that CD57int NK cells make significant amounts of IFN-γ after stimulation with high dose IL-12/IL-18 but respond less robustly to low concentration cytokines and vaccine antigens, suggesting that they may fail to compete effectively with CD57- NK cells when cytokines are limiting.

An area of increasing concern in industrialised countries is the burden of infectious disease and poor response to vaccination in the elderly population [44]. Whilst ageing in the innate immune system, including age-associated changes in the composition, phenotype and function of circulating NK cells, is being linked to increased susceptibility to *de novo* viral and bacterial infections [45], deterioration of antigen-specific memory responses and reduced responsiveness to vaccination with increasing age tends to be attributed to narrowing of the T cell repertoire and functional senescence of the T cell pool [46,47]. Our data suggest, however, that these two components of immune ageing may interact; deteriorating CD4+ T cell responses will limit the availability of IL-2 to drive NK cell responses whilst, at the same time, the proportion of CD57- NK cells able to respond to IL-2 will decrease. We predict, therefore, that vaccination-induced NK cell IFN-γ responses could decline with increasing age, potentially contributing to reduced vaccine efficacy in elderly populations.

In addition, subclinical human cytomegalovirus (HCMV) infections may potentiate the functional differentiation and senescence of NK cells [33-36]. Given that at least 40% of the world population is HCMV seropositive (HCMV+), and prevalence can exceed 95% in some African and Asian populations [48], HCMV exposure may contribute significantly to poor vaccine efficacy at a population level. This hypothesis is the focus of this thesis and is explored explicitly in the following chapter. Other disease states associated with acquisition or loss of

CD57 expression by NK cell populations, and the functional or clinical significance of these changes, have been reviewed by us and are included as Appendix I [8].

In light of my interest in comparing NK cell responses during vaccine recall responses between HCMV uninfected (HCMV-) and HCMV+, it was decided that all future analyses should be performed on the total NK cell population in the first instance (as in Section B and published in White *et al*) rather than the CD56dimCD57- subset (as in some preliminary work in Section A). While focusing on CD56dimCD57- NK cells does indeed improve sensitivity for the detection of NK cell IFN-γ responses, as they are the major responders (Figure 13A-B) , doing so limits the ability to detect differences in NK cell responses between individuals that may due to a re-distribution of CD56/CD57-defined NK cell subsets.

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Chapter 4

Impaired NK cell responses to pertussis and H1N1 influenza vaccine antigens in human cytomegalovirus-infected individuals

The work presented in this chapter is adapted and extended from Nielsen *et al* 2015 ([1], Appendix X).

4.1 Introduction

The natural killer (NK) cell population comprises of a heterogeneous group of cells and the data presented in Chapter 3 (adapted from [2]) clearly illustrates that this heterogeneity has functional consequences during recall responses to vaccines. As CD56dim NK cells gain CD57, maturing from CD56bright to CD56dimCD57- to CD56dimCD57int to CD56dimCD57+, they show decreased responsiveness to exogenous cytokine stimulation (reviewed in [3]). As discussed in Chapter 3, this acquisition of CD57 is associated with both lower CD25 upregulation (the high affinity IL-2 receptor) and IFN-γ production during re-stimulation with vaccine antigens [2]. The major contributors to the IFN-γ response are therefore the CD56bright and CD56dimCD57- subsets. In contrast, degranulation responses are maintained, regardless of CD57 expression.

I would therefore anticipate that factors that influence the proportion of NK cells expressing CD57 would also affect the ability of the total NK cell population to participate in recall responses to vaccine antigens. Ageing is one process associated with a skewing towards the CD56dimCD57+ phenotype, but human cytomegalovirus (HCMV) infection also drives profound changes in the NK cell repertoire (reviewed in [3]).

HCMV infection is strongly associated with a preferential expansion of the CD56dimCD57+NKG2C+ NK cell subset (see Chapter 1 [4-6]). These CD56dimCD57+NKG2C+ NK cells respond poorly to stimulation with pro-inflammatory cytokines such as IL-12 and IL-18. Despite this, these cells are not functionally exhausted as they can respond robustly to direct contact activation, through crosslinking CD16 crosslinking (e.g. by IgG) or natural cytotoxicity receptors (NCRs), to degranulate and secrete cytokines such as IFN-γ and TNF-α [6,7].

These observations imply that, in the context of secondary exposure to a vaccine antigen, NK cells from HCMV-seropositive (HCMV+) individuals may be able to effectively mediate

antibody-dependent cellular cytotoxicity (ADCC) after crosslinking of CD16 by antigen-bound IgG [5,7,8], but may respond poorly to inflammatory cytokines (reviewed in [3]). Specifically, the expanded CD56dimCD57+NKG2C+ subset may be less sensitive to IL-12 and IL-18 from dendritic cells and macrophages, but also to cytokine signals from the adaptive arm of the immune response such as IL-2 from antigen-specific memory CD4+ T cells [2,9].

The characterisation of HCMV-driven changes to the NK cell repertoire has been largely based on studies of haematopoietic stem cell or solid organ transplantation [5,10,11], and follow-up of these patients over time is lacking, in terms of susceptibility to infection or response to vaccination. Additionally, these patients likely represent outliers following the biological trauma of transplantation, and it would be of interest to confirm findings from these studies in HCMV+ healthy adults.

Consequently, the true functional significance of HCMV-driven phenotypic changes to the immune system is poorly understood. Even in the T cell or humoral immunity fields, where the interaction between HCMV serostatus and vaccination outcomes have been more explicitly investigated, the conclusions have been inconsistent. Some studies have reported impaired vaccine responses in HCMV+ donors [12-17], whereas others have found no differences between HCMV+ and HCMV-seronegative (HCMV-) donors ([18-21], as detailed in Chapter 1). More recently, it has even been proposed that HCMV infection may play a beneficial role, by inducing low levels of immune activation that may help to prime responses to heterologous infections (see extended discussion in Chapter 6, [22,23]). The impact of HCMV-driven immune differentiation thus remains unclear.

The aim of this body of work is therefore to compare NK cell responses to vaccine antigens previously encountered during immunisation (*Bordetella pertussis*) or during natural infection (H1N1 influenza virus) in HCMV- and HCMV+ individuals. *B pertussis* is a gram-negative

bacterium that causes whooping cough, a highly contagious respiratory disease. Pertussis immunisation has been included in childhood vaccination programmes in the UK since the 1950s, first with a whole cell preparation before changing to acellular vaccines in 2004. The H1N1 influenza strain used in this study was isolated in 2009 and had been in circulation since then. Both pathogens are of public health concern worldwide [24,25].

4.2 Methods

4.2.1 Study subjects

Volunteers (*n* = 152) were recruited from among staff and students at LSHTM during 2013 (*n* = 52) and 2014 (*n* = 100). All subjects gave fully informed, written consent and the study was approved by the LSHTM Ethics Committee (references # 6237 and # 6324, Appendices IV and XI, respectively). Each subject provided a single 50ml venous blood sample and their reported vaccination history was recorded.

4.2.2 Plasma dialysis and IgG depletion

For experiments that included cell stimulation in the absence of antibody, pooled human AB plasma was IgG-depleted using a protein G Sepharose column (GE Life Sciences). Briefly, AB plasma was diluted 1:1 in complete medium and dialysed in sterile PBS using 10KMWCO (10,000 kD molecular weight cut-off) Slide-a-Lyzer dialysis cassettes (Thermo Scientific) on a stirrer at 4°C, first for four hours and then with fresh PBS overnight. Plasma requiring IgG depletion was then passed through the protein G Sepharose column (GE LifeSciences), as per manufacturer's instructions, twice, using PBS as the buffer solution. Absence of IgG in the column exudate was confirmed using a total IgG ELISA, as described below (Figure 16). Dialysed non-IgG-depleted plasma and dialysed IgG-depleted plasma were then stored in aliquots at -80°C until use.

4.2.3 ELISAs

Total IgG ELISAs were performed with dialysed IgG-depleted and dialysed non-IgG-depleted AB plasma to confirm IgG depletion. AB plasma was also tested IgG antibodies to pertussis toxin (PT; NIBSC: JNIH-5) and to formalin-inactivated whole H1N1 influenza virus (influenza A/California/7/2009(H1N1)v(NYMC-X179A); H1N1; NIBSC: 09/146, Appendix XII). Plates were read at 492nm (Dynex Technologies, MRX TC Revelation Microtiter Plate Reader).

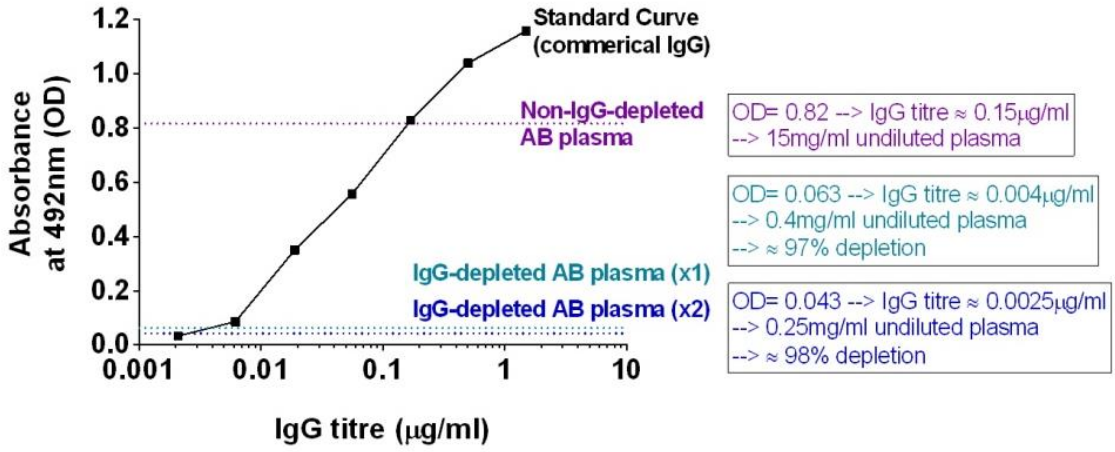

Figure 16. Confirmation of IgG depletion from pooled AB plasma. Pooled AB plasma was dialysed and passed twice over a protein G sepharose column. A total IgG ELISA was used to confirm >95% reduction in IgG from the pooled AB plasma after the first (x1) and second (x2) depletions in comparison to the dialysed but non-depleted pooled AB plasma using interpolation from a standard curve constructed with use of commercial IgG. Dialysed plasma was diluted 1:100,000 for the ELISA. Data are from a single experiment but similar results were obtained with pilot IgG depletions.

For autologous plasma, samples were collected from heparinised whole blood and stored at -80°C until use. Autologous plasma was tested for anti-HCMV IgG, to determine HCMV serostatus, as well as anti-pertussis toxin (PT) and anti-H1N1 IgG.

4.2.3.1 Total IgG ELISA

Total IgG ELISAs were performed to compare dialysed non-IgG-depleted AB plasma and dialysed IgG-depleted AB plasma, using a standardised and validated in-house protocol developed by the Drakeley group at LSHTM (SOP: Detection of Total IgG, edition 003, 01/11/2013). Briefly, this ELISA uses anti-human IgG Fab (Sigma, 15260) as the coating antigen, with goat anti-human IgG (whole molecule) peroxidise (Sigma A8667) as the secondary antibody, and SigmaFast OPD (Sigma) as the substrate. Dialysed plasma was diluted 1:100,000 for the ELISA. The standard curve was produced using commercial IgG (Sigma, I2511) and used to verify IgG-depletion had resulted in a >95% reduction in AB plasma IgG titres (Figure 16).

4.2.3.2 Anti-PT IgG ELISA

The following in-house anti-PT ELISA was adapted from the above protocol for measuring total IgG.

Pertussis toxin was resuspended in 500µl RPMI PSG to give a concentration of 20,000IU/ml and stored in aliquots at -80°C prior to use. Stocks were then diluted 1:100 in coating buffer (Table I, Chapter 2). 96-well flat-bottomed Nunc Immunlon Maxisorp plates were coated overnight with 50µl/well PT coating solution at 4°C. Plates were then washed with PBS/Tween wash solution (0.05%), blotted dry, and blocked with 150µl/well blocking solution (1% skimmed milk powder in PBS/Tween wash solution) for one hour at room temperature. Plates were washed and blotted again before adding 50µl/well of AB or autologous sample plasma (diluted 1:100 in blocking solution) or positive control pertussis antiserum (NIBSC, 06/140) of known anti-PT IgG titre (titrated in blocking solution to give standard curve). All samples and standards were run

in duplicate. Plates were incubated for two hours at 37°C, then washed and dried. A goat HRP-conjugated anti-human IgG (Sigma, A8667) was used as a secondary antibody, diluted 1:5000 in wash solution, with a one-hour incubation at room temperature. Plates were washed and blotted again before adding 100µl/well SigmaFast OPD and incubated for 15 minutes in the dark at room temperature. Reactions were stopped by adding 25µl/well 2M H_2SO_4.

Standard curves were generated using the pertussis reference antiserum titration by plotting dilution against OD on a semi-log curve and calculating the equation of the straight section of the curve. This equation was then rearranged to allow calculation of IgG titres of samples (x) based on OD (y): x = 10^((y-intercept)/slope). If the mean OD of the duplicates of an autologous sample plasma did not fall within the straight line of the standard curve, the ELISA was repeated at a higher or lower dilution, as appropriate, to allow interpolation from the standard curve. The pooled AB plasma used for *in vitro* assays contained 6.8 IU/ml anti-PT IgG.

4.2.3.3 Anti-H1N1 IgG ELISA

An in-house anti-H1N1 IgG ELISA was developed by Martin Goodier, based on my anti-PT IgG ELISA protocol. ELISAs were performed by Martin Goodier and Chiara Lusa.

Briefly, inactivated whole virus H1N1 was used as the coating antigen, with goat anti-human IgG (whole molecule) peroxidase (Sigma A8667) as the secondary antibody, and SigmaFast OPD (Sigma) as the substrate. IgG concentrations were calculated by interpolation from a standard curve which was produced using plasma from a donor with high titres of antibodies to H1N1 influenza (IgG concentration expressed in Arbitrary ELISA Units, AEU; [26]). The pooled AB plasma used for *in vitro* assays contained 273.8 AEU/ml anti-H1N1 IgG.

4.2.3.4 Anti-HCMV IgG ELISA

HCMV infection status was determined by HCMV IgG ELISA (BioKit) using donor plasma diluted 1:100, as per manufacturer's instructions. Donors were consequently determined to be either HCMV-seronegative (HCMV-) or HCMV-seropositive (HCMV+) based on a threshold of 0.25IU/ml anti-HCMV IgG, interpolating from the kit's standard curve. For HCMV+ donors with anti-HCMV IgG titres outside the standard curve, the ELISA was repeated diluting 1:500, 1:1000, or 1:2000 as necessary. In contrast to our in-house ELISAs, this kit uses rabbit anti-human IgG as a secondary antibody and TMB (3,3',5,5'-tetramethylbenzidine) dissolved in DMSO as a substrate. Plates were therefore read at 450nm.

4.2.3.5 Anti-EBV IgG ELISA

Epstein Barr virus (EBV) serostatus was determined by anti-Epstein Barr virus nuclear antigen 1 (EBNA-1) IgG ELISA (Euroimmun) using donor plasma diluted 1:100, as per manufacturer's instructions. TMB/ hydrogen peroxide was used as a substrate and plates were read at 450nm.

4.2.4 IL-2 ELISPOTs

Production of IL-2 in response to killed whole cell pertussis was analysed using an ELISPOT for human IL-2 (Mabtech), as per manufacturer instructions. Briefly, PVDF-membrane plates (MAIPSWU, Millipore) were coated with anti-IL-2 capture antibody overnight at 4°C. After washing plates in PBS, peripheral blood mononuclear cells (PBMC, $2.5x10^5$/well) were incubated with complete medium alone or 0.1IU/ml killed whole cell pertussis (NIBSC: 88/522, Appendix VII), supplemented with 10% FCS, for 18 hours at 37°C. During pilot experiments, anti-CD3 was used as a positive control (2µg/ml, Mabtech), consistently resulting in responses 'too numerous to count' (TNTC; data not shown). Plates were then washed with PBS, incubated with the biotinylated monoclonal detection antibody for two hours, washed with PBS, incubated with streptavidin-ALP (alkaline phosphastase) for two hours, washed again with PBS, and incubated with BCIP/NBT (Mabtech) as a substrate until distinct spots appeared

(routinely 10 minutes), all at room temperature. After drying, plates were imaged and counted with an AID ELISpot Reader. Count settings were standardised across plates and days, using a threshold of 100 pixels as the minimum size for a spot to be counted; this increased the sensitivity of the assay above the default setting (50 pixels). The number of spots was reported per million cells. Measurements of cytokine activity were also taken, as indicated by the size of the spot (arbitrary units). All samples were analysed in triplicate and a mean value was used for analysis.

4.2.5 *NKG2C* genotyping

DNA was extracted from whole blood using a Wizard genomic DNA extraction kit (Promega). Donors were then genotyped for *NKG2C* using touch-down PCR (Phusion High Fidelity PCR kits; New England Biolabs) as optimised in-house by Adriana Goncalves and described previously [27,28]. Two sets of primers (Table III) were used to generate a 200bp fragment for the wild type gene and/ or a 411bp fragment for a *NKG2C* deletion. One hundred ng of DNA was routinely used per sample for the PCR reaction with the following conditions: initial denaturation at 95°C for three minutes; ten cycles of denaturation at 94°C for 30 seconds then annealing starting at 65°C for 30 seconds (then decreasing to 55°C, dropping 1°C per cycle) and extension at 72°C for 30 seconds; followed by 26 cycles of denaturation at 94°C for 30 seconds, then annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. PCR products were separated and identified using agarose gel electrophoresis on a 1.5% agarose TBE (Tris/Borate/EDTA) gel, running at 90V for one hour.

Table III. Primers for *NKG2C* PCR. _F denotes forward primers and _R denotes reverse primers.

Allele	**Primer**	**Primer Sequence**
Wild type *NKG2C*	NKG2C200_F	5'- AGTGTGGATCTTCAATGATA-3
	NKG2C200_R	5’-TTTAGTAATTGTGTGCATCCT-3’
NKG2C deletion	BREAK411_F	5'ACTCGGATTTCTATTTGATGC3'
	BREAK411_R	5'ACAAGTGATGTATAAGAAAAAG3'

4.2.6 PBMC preparation and cell culture

PBMC were isolated, cryopreserved, and thawed as described in Chapter 2.

PBMCs were cultured for 18 hours at 37°C at approximately 2×10^5/well in 96-well U-bottom plates (Nunc) in complete medium with or without low concentration of cytokines (LCC; 12.5 pg/ml recombinant human [rh] IL-12 [PeproTech] plus 10 ng/ml rhIL-18 [MBL]); high concentration of cytokines (HCC; 5 ng/ml rhIL-12 plus 50 ng/ml rhIL-18); rat anti-IL-2 (3 µg/ml; BD Biosciences); rat IgG2A isotype control (3 µg/ml; BD Biosciences); 1 µg/ml formalin-inactivated whole H1N1 influenza virus; 1 IU/ml killed whole-cell *B. pertussis* (pertussis, as above; NIBSC: 88/522, Appendix VII); or MHC class I–deficient K562 target cells (effector:target [E:T] ratio 2:1). GolgiStop (containing Monensin, 1/1500 concentration; BD Biosciences) and GolgiPlug (containing brefeldin A, 1/1000 final concentration; BD Biosciences) were added after 15 hours. Anti-CD107a antibody (A488-conjugated; BD Biosciences) was included in the medium for the entirety of cell culture. Dialysed plasma samples were used for all cell culture assays where read-outs were being compared to IgG-depleted plasma conditions. K562 cell line cultures were maintained in complete medium (see Chapter 2) supplemented with 10% FCS, splitting 1:10 every 2-3 days.

For activation via crosslinking, as outlined in Chapter 2, 96-well flat-bottom plates (Nunc) were coated with 20µg/ml anti-human CD16 (BD Biosciences), anti-NKp30 and anti-NKp46 (R&D Systems), or isotype-matched control antibodies (mIgG1k, BD Biosciences; or IgGA and IgG2B; R&D Systems) overnight at 4°C. Cells were plated out at approximately 4×10^5 PBMCs/well, which had been incubated overnight at 37°C in 10% AB plasma with or without 50-100 IU/ml IL-2 (PeproTech). Anti–CD107a-A488 was added at the beginning of culture, and all assays were performed in complete medium supplemented with 10% AB plasma. Cells were

harvested after 5 hours and then centrifuged at 754g for five minutes and washed prior to Staining as per standard protocol (Chapter 2).

T cell assays were synchronised with crosslinking experiments and PBMC were therefore incubated overnight at 37°C in 10% AB plasma in 5ml polypropylene round-bottom tubes (Falcon). Cells were then transferred to 96-well round-bottomed plates, approximately 4×10^5 PBMCs/well, and stimulated for 5 hours in medium alone, 1 μg/ml formalin-inactivated whole H1N1 influenza virus, or 1 IU/ml killed whole-cell *B. Pertussis*, or PMA/ ionomycin as a positive control. GolgiStop and GolgiPlug were added after one hour, as described above. Cells were harvested and stained as per standard protocol.

4.2.6 Flow cytometry

Responses of NK cells and T cells were assessed as described previously and outlined in Chapter 2. The following reagents were used: anti-CD3-V500, anti-CD56-phycoerythrin (PE)-Cy7, anti-IFN-*γ*-APC, anti-IFN-*γ*-PE, anti-CD4-PE, anti-IL-2-APC, anti-CD45RA-APC-H7, anti-CD107a-FITC (all BD Biosciences); anti-CD25-PerCP-Cy5.5, anti-IFN-*γ*-e780, anti-CD16-APC, anti-CD16-e780, anti-CD8-PECy5, anti-ILT2-PE, anti-CD161-PerCP-Cy5.5, anti-CD4-e450, anti-CCR7-APC, anti-IL-18R*α*-PE, and anti-CD57-e450 (all e-Biosciences); anti-NKG2C-PE, anti-NKG2C-APC (both R&D Systems), and anti-NKG2A-FITC (Miltenyi). Anti-IL-12Rβ2 monoclonal antibody was obtained from R&D Systems and conjugated to PE-Cy5 using an Easylink PE/Cy5® Conjugation Kit (Abcam). Compensation controls were prepared at the time of cell staining using BD Biosciences CompBeads.

4.2.7 Statistical analyses

Figures showing 52 donors represent data from 18 experiments (2-3 donors per experiment) and figures showing 152 donors represent data from 24 experiments (16-20 donors per experiment for additional 100 donors); sample sizes and number of experiments represented

in each figure are described in legends. Flow cytometry and statistical analyses were performed as described in Chapter 2 and also as detailed in figure legends.

For flow cytometry experiments, cell populations with fewer than 100 cells were excluded from the analyses and thus the number of samples included varies between figures, depending on the cell population being analysed. This is incorporated in the statistics, though note that the sample sizes stated in figure legends refer to the total number of donors included in the assay prior to exclusions.

Mann–Whitney tests were used to compare responses between HCMV- and HCMV+ donors, and linear regression (STATA) was used to adjust for sex and age. Unless otherwise stated, statistical tests were one-sided. ****$p \leq 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

4.3 Results

4.3.1 Donor characterisation

Subject characteristics are summarised in Table IV. Subjects (*n* = 152) ranged in age from 20-77-years (media*n* = 33-years). Fifty-five subjects (36%) were found to be HCMV+, consistent with what has previously been published on UK populations for the age range sampled (Figure 17A). As seroprevalence was consistent between the two recruitment cohorts (2013- 37%; 2014- 36%), all analyses were pooled. Anti-HCMV IgG titre increased significantly with increasing age (R^2 = 0.248, *p* = 0.0001; Figure 17B) but age did not differ significantly between HCMV+ and HCMV- donors (two-tailed Mann-Whitney test, *p* = 0.561; Table IV). As the proportion of female and male donors differed between the HCMV- and HCMV+ groups subsequent analyses were adjusted for sex (Tables IV-V).

Table IV. Donor characteristics. Donors were classified as human cytomegalovirus seronegative (HCMV-) and seropositive (HCMV+) by anti-HCMV IgG ELISA, using 0.25IU/ml as the cut-off as per manufacturer's instructions. *NKG2C* genotype (*NKG2C*$^{+/+}$, *NKG2C*$^{+/-}$, *NKG2C*$^{-/-}$) was determined by PCR. IgG antibody titres against pertussis toxin (PT) and H1N1 were calculated with in-house ELISAs from interpolation of a reference serum or high titre donor standard curve, respectively. Epstein Barr (EBV) serostatus was determined using an anti-EBNA-1 IgG ELISA, as per manufacturer's instructions.

	HCMV- **(*n* = 97)**	**HCMV+** **(*n* = 55)**
Median Age, Years (range)	32 (20-70)	35 (21-77)
Female *n* (%)	73 (75)	32 (58)
***NKG2C* Genotype** $^{+/+}$, $^{+/-}$, $^{-/-}$ ***n* (%)**	67/24/2 (72/26/2)	35/17/2 (65/31/4)
***NKG2C*$^{-}$ Haplotype Frequency (%)**	15.0	19.4
anti-HCMV IgG titre IU/ml median (range)	< 0.25	394.2 (31.1-4411.6)
anti-PT IgG titre IU/ml median (range)	6.7 (0.5-139.3)	5.0 (0.8-179.9)
anti-H1N1 IgG titre AEU*/ml median (range)	214.6 (80.7-953.2)	190.1 (90.2-522.7)
EBV-seropositive *n* (%)	82 (85)	52 (95)

* arbitrary ELISA units, see Methods.

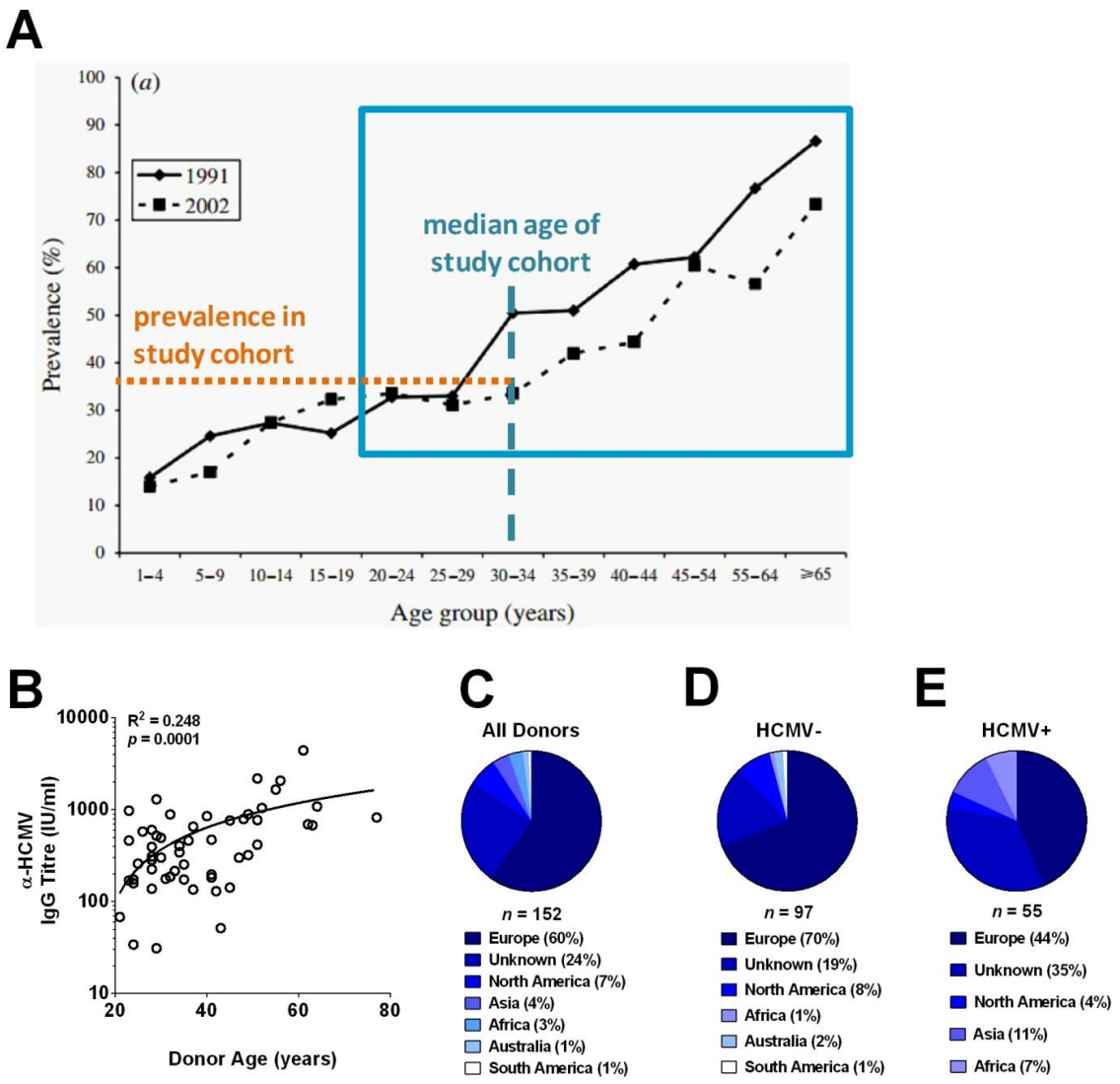

Figure 17. HCMV prevalence is consistent with previous UK observations and anti-HCMV IgG titres increase with age in HCMV+ donors. HCMV prevalence in study blood donors is consistent with previously published data from the UK, adapted from Vyse *et al* 2009 [29]: the orange dashed line indicates HCMV seroprevalence in my study cohort (36%), while the blue dashed line indicates median age (33 years), and the blue box corresponds to the age range (20-77 years) **(A)**. Within the HCMV+ donors, there is a significant association between age and anti-HCMV IgG titre calculated by ELISA, as determined by bivariate nonlinear regression. Each data point represents one donor, *n* = 55. Data are from two experiments **(B)**. The majority of study donors were born in Europe **(C)**, although the proportion is potentially higher in the HCMV- **(D)** than HCMV+ **(E)** group.

There was some evidence to suggest that HCMV+ donors were less likely to have been born in the Europe or North America than HCMV- donors (Figures 17C-E), but data on country of birth was not available for a substantial proportion of donors, precluding more detailed further analyses. Similarly, no data on ethnicity was available.

Cells from all 152 subjects were analysed for responses to pertussis. One hundred and fourteen donors (75.0%) confirmed that they had been vaccinated against pertussis but a minority of donors reported that they had not been vaccinated against pertussis (*n* = 13; 8.6%) or were unsure of their vaccination status (*n* = 25; 16.4%). However, the proportions of these individuals did not differ between the HCMV+ and HCMV- groups (Chi square test, *p* = 0.640; Figure 18A). Furthermore, their antibody titres did not suggest a difference in vaccination history: vaccinated individuals had a median anti-PT IgG titre of 6.5IU/ml as compared to 4.8IU/ml in unvaccinated or unsure individuals (two-tailed Mann-Whitney test, *p* = 0.215). The median anti-PT IgG titre was higher among HCMV- donors than among HCMV+ donors, but this difference was not statistically significant either (6.7 IU/ml vs 5.0 IU/ml, two-tailed Mann-Whitney, *p* = 0.078; Figure 18B).

All donors analysed for responses to vaccine H1N1 influenza (*n* = 52) confirmed no previous seasonal influenza vaccination to H1N1, i.e. only natural environmental exposure. Median anti-H1N1 IgG titres were higher among HCMV- donors (204.1 AEU/ml) than among HCMV+ donors (187.2 AEU/ml), although again this difference was not statistically significant (two-tailed Mann-Whitney, *p* = 0.135; Figure 18C).

All donors were also tested for Epstein Barr virus (EBV) infection, using an anti-Epstein Barr virus nuclear antigen 1 (EBNA-1) IgG ELISA. Although the proportion of EBV+ individuals was higher in the HCMV+ group as compared to HCMV- (95% and 85%, respectively), this difference was not significant (two-tailed Fisher's exact test, *p* = 0.073; Table IV).

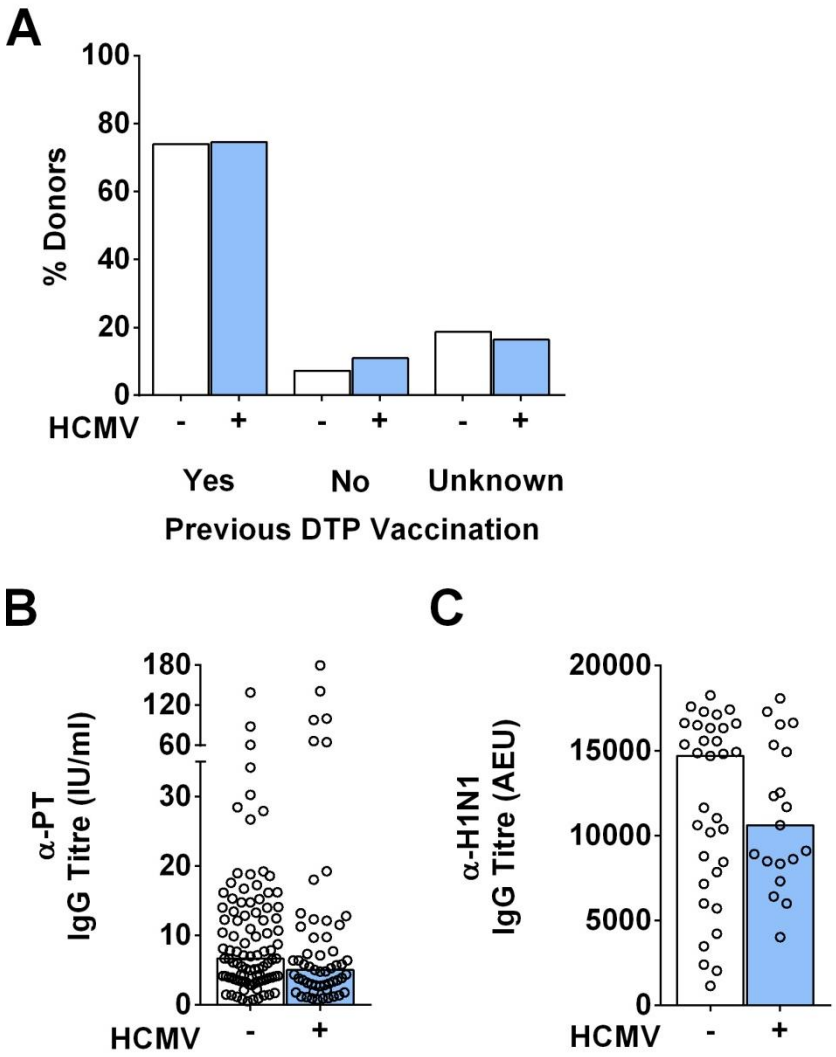

Figure 18. No difference in self-reported DTP vaccination history between HCMV- and HCMV+ donors. Self-reported vaccination history with DTP (diphtheria-tetanus-pertussis) was not significantly different between HCMV- (*n* = 97) and HCMV+ (*n* = 55) donors **(A)**, nor were anti-pertussis toxin (PT) **(B)** or anti-H1N1 **(C)** IgG titres as determined by in-house ELISAs. Each data point represents one donor and bars denote medians **(B-C)**. Comparisons were performed with two-tailed Mann Whitney tests; no differences were significant **(B-C)**. Data are from 4 experiments **(B)** or 5 experiments **(C)**.

4.3.2 Antibody and antigen-specific IL-2 drive NK cell responses to pertussis and H1N1 influenza virus

PBMC from 100 donors were stimulated overnight with pertussis (Figure 19B-D) and NK cell responses were measured by flow cytometry (Figure 19A). Significant induction of CD25 and IFN-γ (Figure 19B-C) and degranulation (as measured by cell surface expression of lysosomal marker CD107a [30]; Figure 19D) was observed in response to pertussis. Analysis of this response by CD56bright and CD56dim subsets reveals that the CD56dim cells respond more robustly to pertussis than do the CD56bright NK cells and are thus the major contributors to the vaccine response, since they are also more numerous than CD56bright cells (Figure 20A-C).

Co-expression of CD25/IFN-γ was markedly attenuated in the presence of a blocking antibody to IL-2 and after depletion of IgG from the plasma used to supplement the culture medium, indicating a role for both memory T cell-derived IL-2 and antigen-antibody complexes in the NK cell IFN-γ response (Figure 19C). By contrast, the degranulation response (CD107a) was dependent upon IgG but not IL-2 (Figure 19D). The observation that neither anti-IL-2 nor IgG depletion completely abrogated the NK cell IFN-γ response suggests that these two signals may synergise for optimal IFN-γ production.

Cells from a subset of subjects (*n* = 16) were also analysed for responses to H1N1 influenza in the context of IL-2 blockade or IgG depletion (Figure 19E-G). As observed with pertussis, statistically significant induction of CD25 (Figure 19E), CD25/IFN-γ (Figure 19F) and CD107a (Figure 19G) was observed in response to re-stimulation with H1N1 antigen, and IL-2 blocking significantly decreased CD25/IFN-γ expression (Figure 19F) whilst IgG depletion inhibited the degranulation (CD107a) response (Figure 19G). Interestingly, and in contrast to the response to pertussis, IgG depletion enhanced IFN-γ production in response to H1N1 and IL-2 blockade slightly decreased degranulation, perhaps indicating competition between these pathways for NK cell activation during influenza responses (Figure 19F).

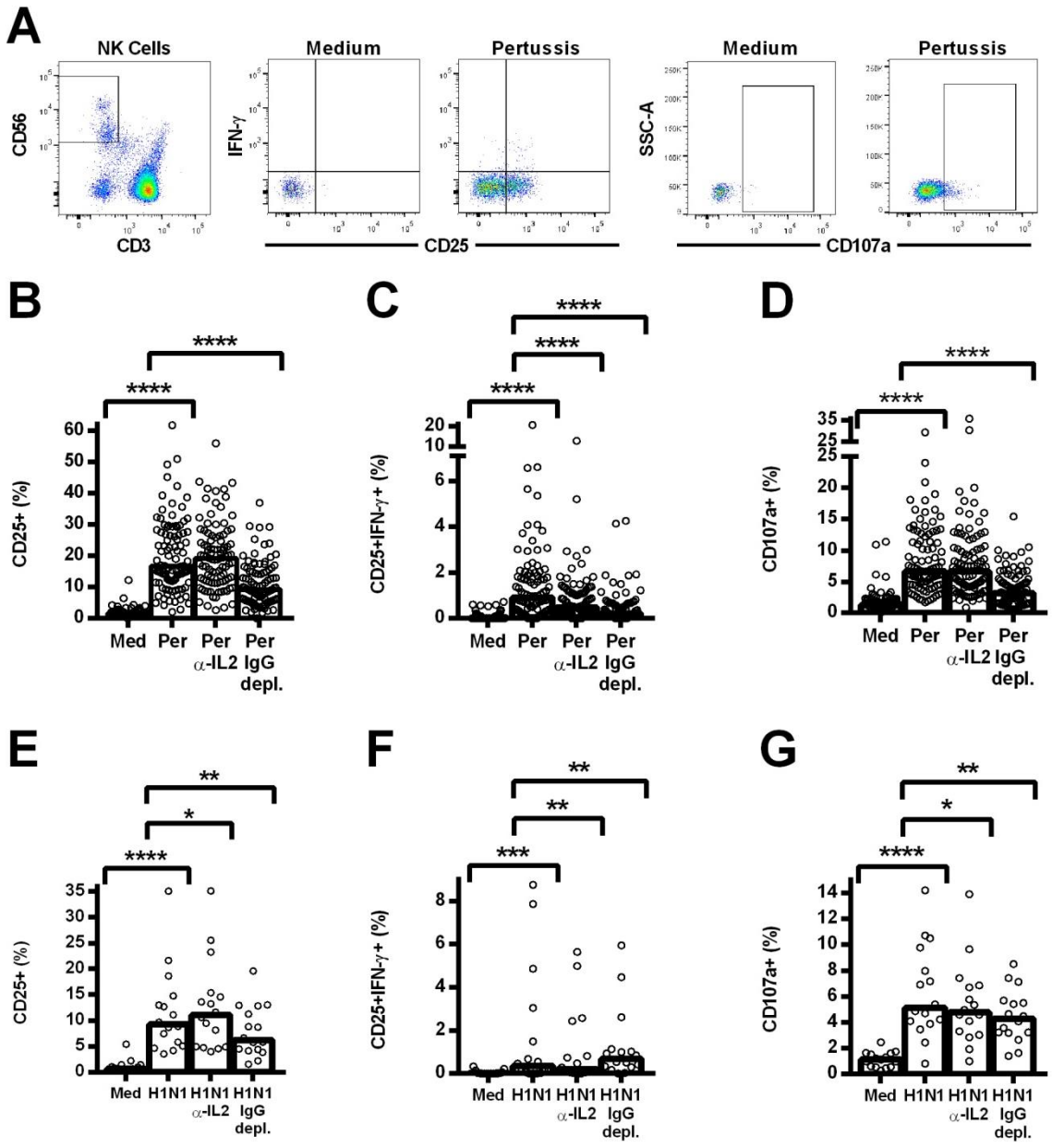

Figure 19. NK cell responses to pertussis and H1N1 are inhibited by IL-2 neutralisation and IgG depletion. PBMC were cultured *in vitro* for 18 hours with medium alone (Med), killed whole cell pertussis (Per), inactivated whole H1N1 influenza virus (H1N1), pertussis or H1N1 with blocking antibody to IL-2 (Per α-IL-2, H1N1 α-IL-2) or pertussis or H1N1 in IgG-depleted plasma (Per IgG depl., H1N1 IgG depl.). The isotype control antibody (IgG2A) for the IL-2 blocking antibody was included in the medium, pertussis, and H1N1 wells. Representative flow cytometry plots show gating of CD3-CD56+ NK cells and expression of CD25, IFN-γ, and CD107a **(A)**. Responses to pertussis **(B-D)** and H1N1 **(E-G)** were measured by the percentage of NK cells expressing CD25 **(B, E)**, co-expressing CD25/IFN-γ **(C, F)**, and expressing CD107a **(D, G)**. Data were analysed using paired, one-tailed Wilcoxon signed-rank tests. **** $p \leq 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Each data point represents one donor, $n = 100$ **(B-D)** or $n = 16$ **(E-G)**, and bar graphs denote medians. Data are either from 6 experiments **(B-D)** or 3 experiments **(E-G)**.

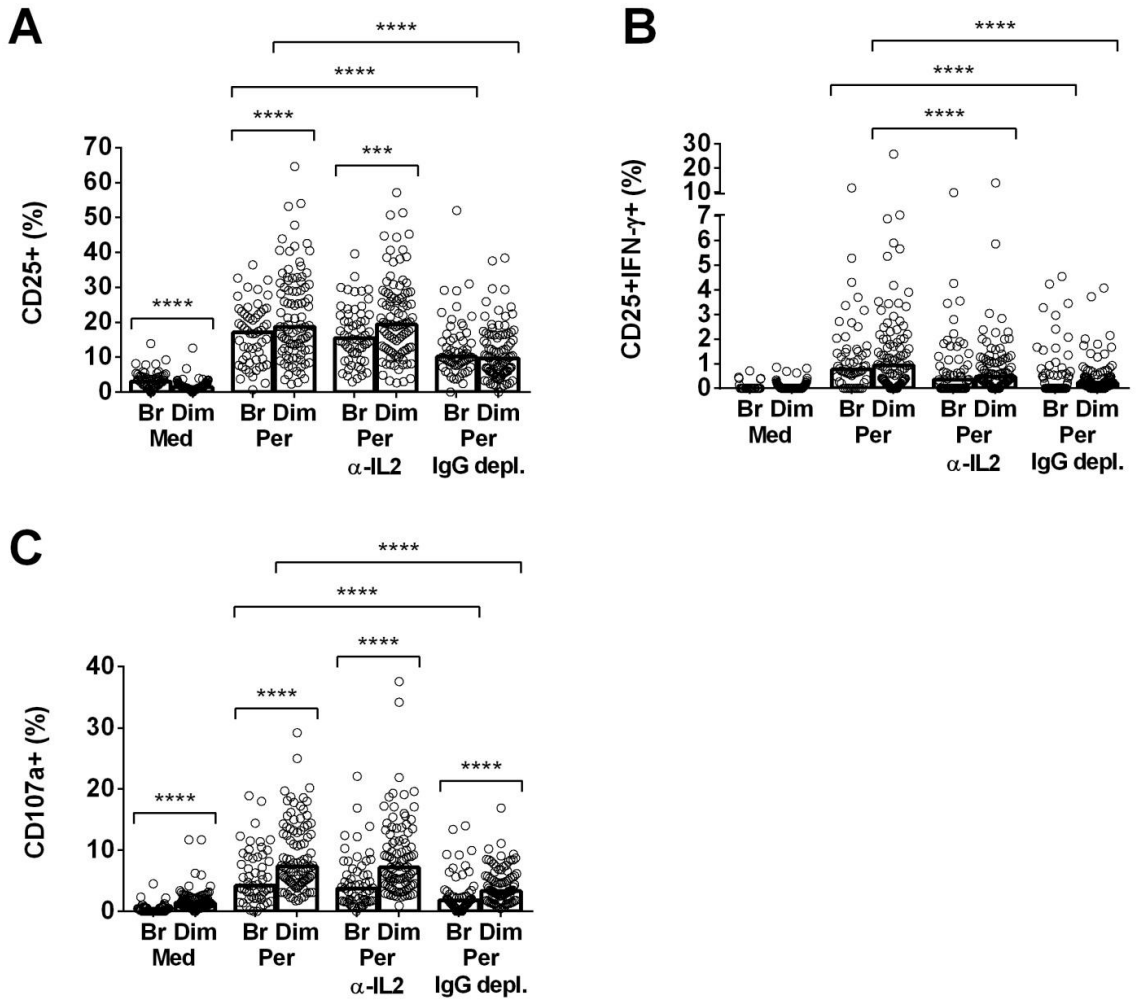

Figure 20. CD56bright and CD56dim NK cell responses are inhibited by IL-2 neutralisation and IgG depletion. PBMC were cultured *in vitro* for 18 hours with medium alone (Med), killed whole cell pertussis (Per), pertussis with blocking antibody to IL-2 (Per α-IL2) or pertussis in IgG-depleted plasma (Per IgG depl.). The isotype control antibody (IgG2A) for the IL-2 blocking antibody was included in the medium and pertussis wells. Responses were measured by the percentage of CD56bright (Br) or CD56dim (Dim) NK cells expressing CD25 **(A)**, co-expressing CD25/IFN-γ **(B)**, and expressing CD107a **(C)**. CD56bright and CD56dim responses were compared to each other for each condition, then to pertussis alone for the IL-2 blocking and IgG depletion conditions. Data were analysed using paired, one-tailed Wilcoxon signed-rank tests. **** $p \leq 0.0001$, *** $p < 0.001$. Each data point represents one donor, $n = 100$, and bar graphs denote medians. Data are from 6 experiments.

4.3.3 HCMV infection is associated with impaired NK cell responses to pertussis and H1N1 influenza virus

NK cell responses to pertussis (*n* = 152) and H1N1 (*n* = 52) were compared between HCMV- and HCMV+ donors (Figure 21). Consistent with prior observations [2,9], responses to pertussis and H1N1 were significantly augmented by low concentrations of cytokines IL-12 and IL-18 (LCC; $p \leq 0.0001$ for all parameters) indicating that *in vitro* accessory cell activation and production of IL-12 and IL-18 (which is essential for IL-2-mediated NK cell activation [9,31,32]) were suboptimal.

Interestingly, in the absence of LCC, pertussis induces stronger NK cell responses than H1N1 whereas in the presence of LCC, H1N1 induces the more robust responses. This may indicate that pertussis induces some IL-12 and IL-18 secretion (such that LCC is redundant in these assays) whereas H1N1 may be a poor inducer of IL-12 and IL-18 but a better inducer of IL-2 or other accessory cytokines. This would be consistent with differences in Toll-like receptor (TLR) signalling by RNA viruses such as influenza (TLR3) and gram-negative bacteria such as pertussis (TLR4) [33-36].

NK cells from both HCMV+ and HCMV- donors responded to pertussis and H1N1 (with or without LCC; Figure 21), however NK cell responses to these two vaccines (whether defined as CD25+, CD25+IFN-γ+, or CD107a+) were significantly lower among HCMV+ donors than among HCMV- donors (Figure 21A,C,E). This was true for both vaccines and all parameters when cells were cultured with LCC, and was also true for the CD25+ responses to H1N1 and all responses to pertussis in the absence of LCC. Additionally, NK cell CD25+, CD25+IFN-γ+ and CD107a+ expression in response to HCC (high concentrations of IL-12 and IL-18) were all significantly higher in HCMV- compared to HCMV+ donors (Figure 21A,C,E).

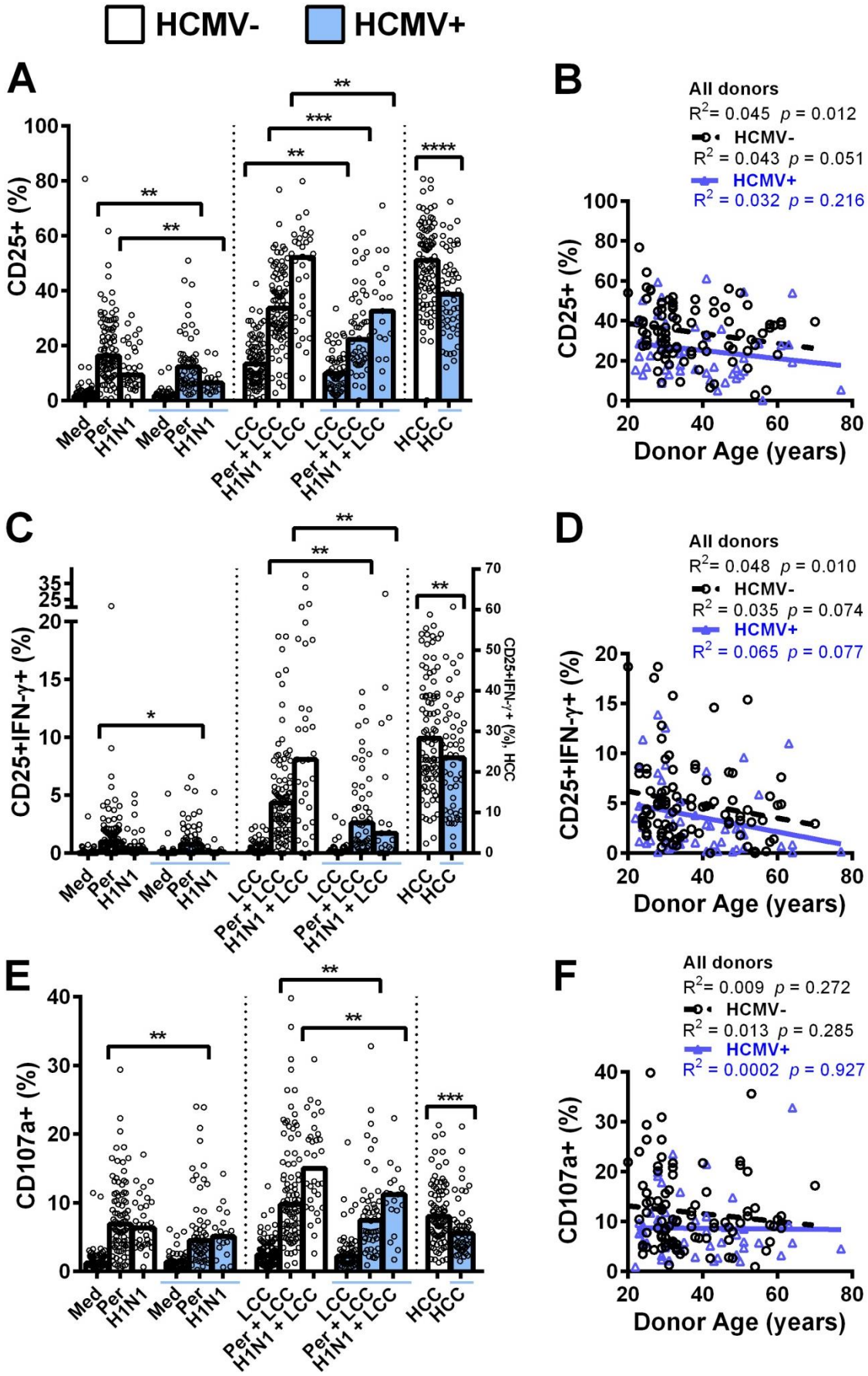

Figure 21. NK cell responses to vaccine antigen are affected by HCMV infection. PBMC were cultured *in vitro* for 18 hours with medium alone (Med), low concentration of cytokines (LCC: 12.5 pg/ml IL-12 and 10ng/ml IL-18), killed whole cell pertussis (Per), inactivated whole H1N1 influenza virus (H1N1), Per + LCC, H1N1 + LCC, or high … **(continued on page 116)**

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… concentration of cytokines (HCC: 5ng/ml IL-12, 50ng/ml IL-18). Donors were stratified into HCMV- and HCMV+ groups. Responses were measured as the percentage of NK cells expressing CD25 **(A-B)**, co-expressing CD25/IFN-γ **(C-D)**, or CD107a **(E-F)**. Data were analysed using, one-tailed Mann-Whitney tests. **** $p \leq 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Bivariate regression of age against responses to Per + LCC was performed for the percentage of NK cells expressing CD25 **(B)** CD25/IFN-γ **(D)**, and CD107a **(F)**. Each data point represents one donor, n = 152 (24 experiments), except for H1N1 and H1N1 + LCC where n = 52 (18 experiments). Bar graphs denote medians.

NB, all antigen stimulations induced statistically significant increases in expression of CD25, CD25/IFN-γ, and CD107a over background (medium alone for pertussis/ H1N1, or LCC for pertussis+LCC /H1N1+LCC; $p < 0.05$ in all cases), except that H1N1 did not induce a significant increase in CD25+IFN-γ+ NK cells in HCMV+ donors ($p = 0.416$).

Analysis of this response by CD56bright and CD56dim subsets reveals that the effect of HCMV status is due almost entirely to an effect within the CD56dim subset (Figure 22). Importantly, *ex vivo* (data not shown) and resting levels of CD25 expression *in vitro* did not differ significantly between HCMV+ and HCMV- donors (Figure 21A), and although there were significant differences in the T cell populations (Figure 23), there was no difference in the potential of T cells from HCMV- and HCMV+ donors to produce IL-2 in response to pertussis antigen (Figure 24). Specifically, HCMV- donors have higher proportions of naïve CD4+ T cells (CD45RA+CCR7+) and lower proportions of effector memory CD4+ T cells (CD45RA-CCR7-), consistent with published reports (Figure 23A; see Chapter 1 [16,37]). While the trend was the same for CD8+ T cells, the differences were not statistically significant (Figure 23B). HCMV- donors also had a substantially higher ratio of CD4+:CD8+ T cells, another illustration of profound changes in the T cell repertoire attributable to HCMV infection (Figure 23C).

Due to cell limitations from the 2013 cohort (used for H1N1 assays, n = 52) I was unable to similarly compare H1N1 responses by ELISPOT, but the IL-2 blocking data suggests a comparable role for T cell-derived IL-2 as in the pertussis responses (Figure 19). There was also no indication from preliminary flow cytometry T cell data that there were any differences in CD4+ T cell IL-2 or T cell IFN-γ responses to pertussis or H1N1, although these analyses were

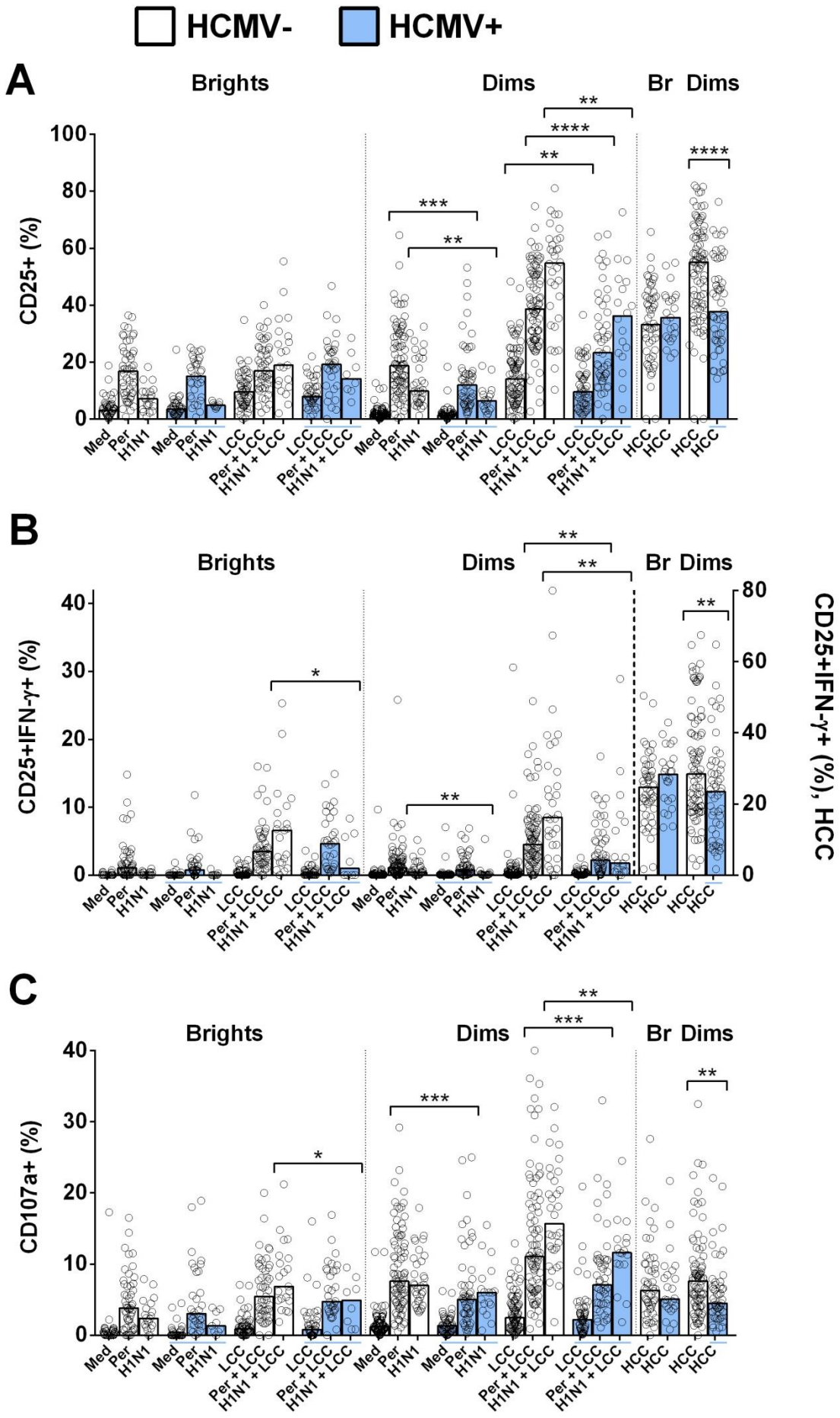

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Figure 22. CD56bright and CD56dim NK cells respond more poorly to vaccine antigens in HCMV+ donors as compared to HCMV- donors. PBMC were cultured *in vitro* for 18 hours with medium alone (Med), low concentration of cytokines (LCC: 12.5pg/ml IL-12 and 10ng/ml IL-18), killed whole cell pertussis (Per), inactivated whole H1N1 influenza virus (H1N1), Per + LCC, H1N1 + LCC, or high concentration of cytokines (HCC: 5ng/ml IL-12, 50ng/ml IL-18). Donors were stratified into HCMV- and HCMV+ groups. Responses were measured as the percentage of CD56bright or CD56dim NK cells expressing CD25 **(A)**, co-expressing CD25/IFN-γ **(B)**, or CD107a **(C)**. Data were analysed using, one-tailed Mann-Whitney tests. **** $p \leq 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Each data point represents one donor, $n = 152$ (24 experiments), except for H1N1 and H1N1 + LCC where $n = 52$ (18 experiments). Bar graphs denote medians.

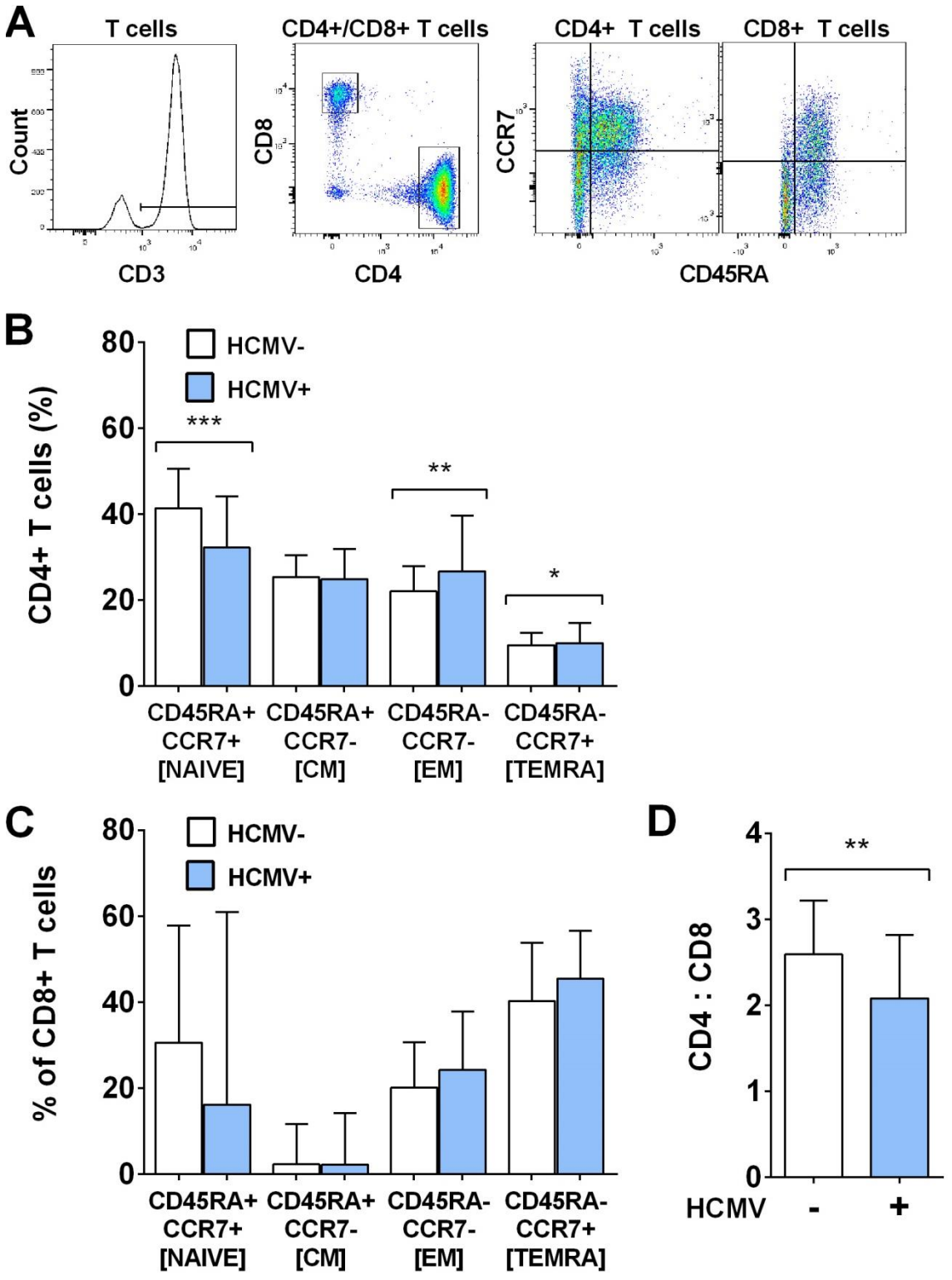

Figure 23. Changes to CD4+ and CD8+ T cell populations in HCMV+ donors. PBMC were analysed *ex vivo* for surface expression of CD3, CD4, CD8, CD45RA and CCR7, as shown by representative flow cytometry plots **(A)**. Proportions of CD4+ **(B)** or CD8+ **(C)** T cells expressing CD45RA/CCR7 [corresponding to central memory (CM), naïve, terminally differentiated effector memory (TEMRA), or effector memory (EM) subsets], or the ratio of CD4:CD8 T cells were compared between HCMV- and HCMV+ donors **(D)** using two-tailed Mann-Whitney tests. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. $n = 152$ (24 experiments). Bars represent medians and lines denote interquartile ranges.

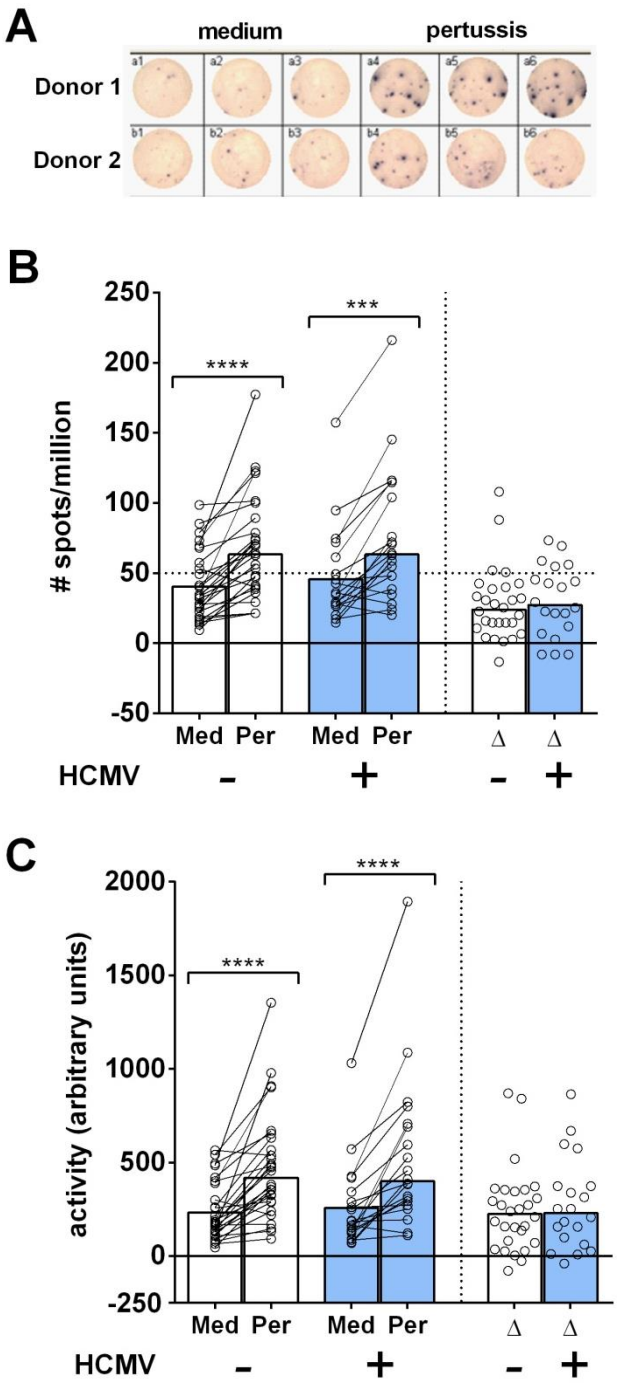

Figure 24. IL-2 production in response to pertussis is equally robust in HCMV- and HCMV+ donors. PBMC were cultured *in vitro* for 18 hours with killed whole cell pertussis (Per) or without (Med; medium) and assessed for IL-2 production using a human IL-2 ELISPOT (ALP) kit (Mabtech), as per manufacturer's instructions. Responses were visualised and analysed on an AID ELISPOT plate reader; an example photograph is shown for two donors **(A)**. Responses were measured in terms of the number of cells producing IL-2 (spots/million, **B**) and the amount of IL-2 produced (activity, **C**). Delta (Δ) values were calculated by subtracting the background response (Med) from the response to pertussis (Per). Responses to pertussis for each donor were compared to medium alone using two-tailed Wilcoxon tests, and responses were compared between HCMV- (-) and HCMV+ (+) donors using two-tailed Mann-Whitney tests. **** $p \leq 0.0001$, *** $p < 0.001$. Each data point represents the mean of three technical replicates for a single donor (n = 48) and bar graphs denote medians. Data are from 3 experiments.

constrained due to high background responses with medium alone (Figure 25). However, analysis of assays I performed with post-vaccination PBMC samples from an influenza vaccination intervention study with these same donors also showed no differences in IL-2 responses to H1N1 between HCMV- and HCMV+ donors (Goodier, Rodríguez-Galán, Lusa, Nielsen *et al*, manuscript accepted). This supports the conclusion by van Leeuwen *et al* that there is no evidence of an association between HCMV-specific T cell expansions and the capacity of an individual to respond to heterologous antigens [38].

In addition to consistently lower NK cell responses to vaccine antigens in HCMV+ individuals, there was a trend for CD25 and CD25/IFN-γ responses to pertussis (with or without LCC) to decline with increasing age (Figure 21B, 21D). This was statistically significant for the cohort as a whole, but not when analysed separately for HCMV- and HCMV+ donors due to decreased power. There was no effect of age on CD107a upregulation, which is consistent with decreased sensitivity to exogenous cytokines but maintained cytotoxicity during normal ageing (reviewed in [39]) and increasing NK cell differentiation [40,41]. Importantly, the effect of HCMV infection on impaired NK cell responses to pertussis and H1N1 is entirely independent of the association between age and NK cell function. In line with this conclusion, adjusting for age by parametric regression did not alter the conclusions of the study (Table V).

Overall, NK cell responses did not differ significantly between males and females although there was a trend for median responses to be higher in women than in men and this reached statistical significance ($p < 0.05$) for the IFN-γ response to pertussis + LCC in HCMV+ donors (Figure 26). As the proportion of female subjects differed between the HCMV- and HCMV+ groups (Table IV), the data in Figure 21 were reanalysed, adjusting for sex as well as age using parametric regression (Table V). After adjustment for sex and age, CD25/IFN-γ and CD107a expression in response to vaccine alone (i.e. without LCC) are no longer significantly different

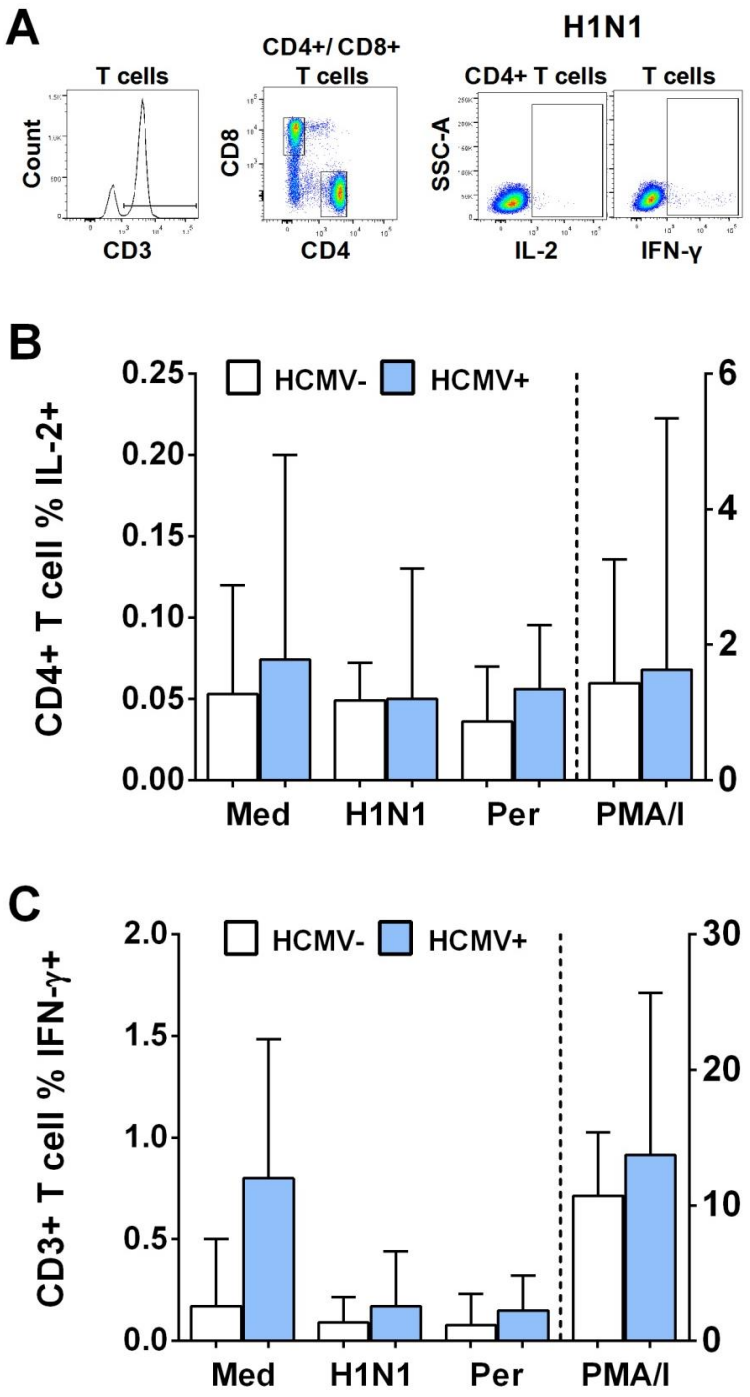

Figure 25. T cell responses as measured by flow cytometry indicate no difference between HCMV- and HCMV+ donor responses to pertussis or H1N1. PBMC were cultured *in vitro* for five hours with medium alone (Med), inactivated whole H1N1 influenza virus (H1N1), killed whole cell pertussis (Per), or PMA and ionomycin (PMA/I) as a positive control. Representative flow cytometry plots from a sample stimulated with H1N1 show gating of CD3+ T cells from the total lymphocyte population, gating of CD4+ and CD8+ T cells, and expression of intracellular IL-2 by the CD4+ T cells, or intracellular IFN-γ by the total T cell population **(A)**. IL-2 responses by CD4+ T cells to Med, H1N1, Per, and PMA/I were compared between HCMV- and HCMV+ donors **(B)**, as were IFN-γ responses by all CD3+ T cells **(C)**. Note that PMA/I responses are plotted on the right-hand axes **(B-C)**. Data were analysed using two-tailed Mann Whitney tests; no differences were significant. Bars represent medians and lines denote interquartile ranges. *n* = 52 (18 experiments).

Table V. NK cell responses to vaccine antigens by HCMV status after adjusting for sex and age. A regression analysis was performed in STATA to adjust for sex and age when comparing NK cell responses to pertussis (-/+low concentration of cytokines [LCC: 12.5pg/ml IL-12 and 10ng/ml IL-18]), H1N1 (-/+ LCC), and high concentration of cytokines (HCC: 5ng/ml IL-12, 50ng/ml IL-18) between HCMV- and HCMV+ donors. The response was quantified by the percentage of total NK cells expressing CD25, CD25/IFN-γ (CD25+IFN-γ+), and CD107a.

Stimulus	Parameter (Total NK cells)	Adjusted for sex and age	
		Effect (95% CI)[1]	*p* value[2]
Pertussis	CD25+	-4.4 (-8.3, -0.5)	0.014
	CD25+IFNγ+	-0.5 (-1.2, 0.3)	0.125
	CD107a+	-1.5 (-3.4, 0.5)	0.071
Pertussis + LCC	CD25+	-8.5 (-13.7, -3.4)	0.001
	CD25+IFNγ+	-1.5 (-2.8, -0.1)	0.020
	CD107a+	-2.9 (-5.5, -0.3)	0.016
H1N1	CD25+	-5.4 (-9.5, -1.3)	0.005
	CD25+IFNγ+	-0.4 (-1.1, 0.4)	0.158
	CD107a+	-1.8 (-3.9, 0.3)	0.049
H1N1 + LCC	CD25+	-12.2 (-22.6, -1.8)	0.011
	CD25+IFNγ+	-5.1 (-10.4, 0.1)	0.027
	CD107a+	-5.1 (-8.9, -1.5)	0.004
HCC	CD25+	-11.3 (-16.7, -6.0)	<0.0001
	CD25+IFNγ+	-6.5 (-11.4, -1.7)	0.005
	CD107a+	-2.1 (-3.5, -0.6)	0.004

[1] Effect (coefficient), with 95% confidence interval, represents the change in the mean percentage of NK cells responding in HCMV+ donors as compared to HCMV- donors.

[2] The *p* value refers to the significance of the difference in response between HCMV- and HCMV+ donors after adjusting for sex and age. Underlined *p* values < 0.05.

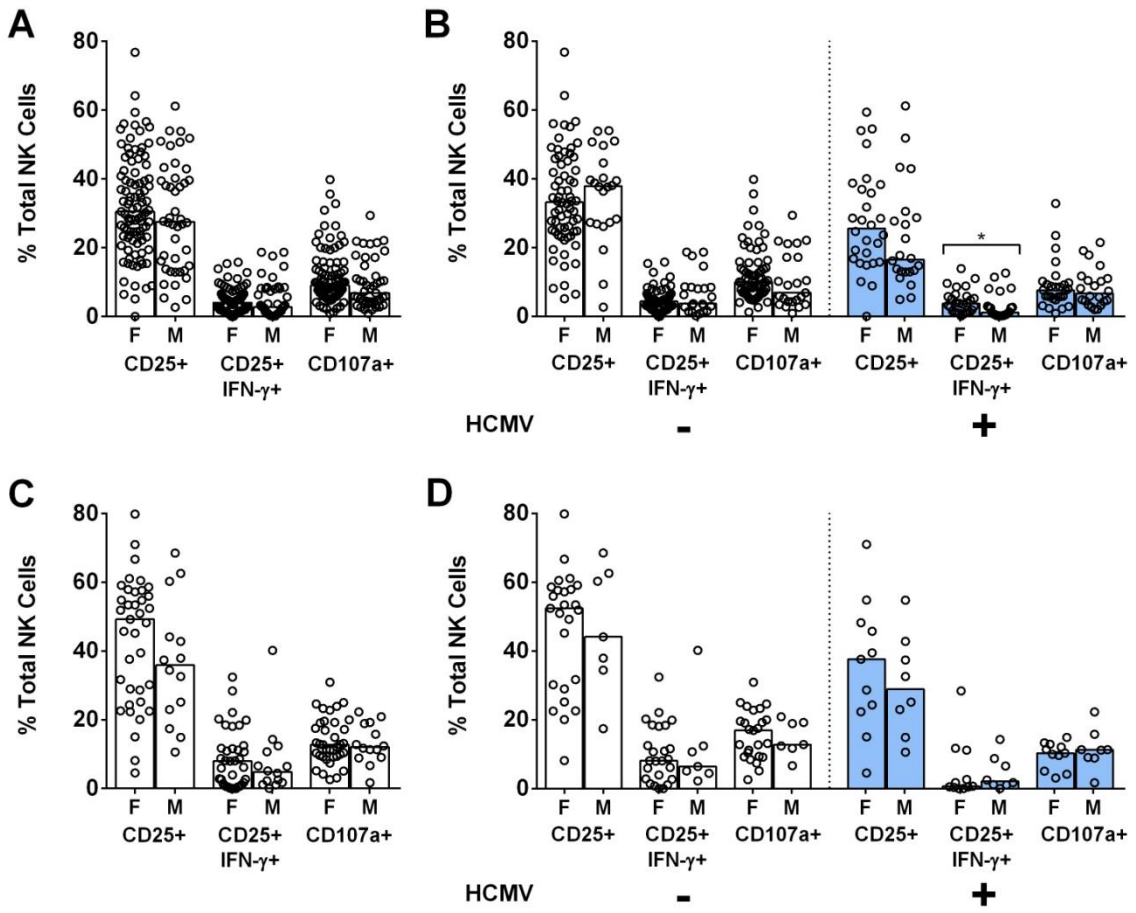

Figure 26. Female donors may have higher NK cell responses to vaccine antigens than male donors. PBMC were cultured *in vitro* for 18 hours with killed whole cell pertussis with a low concentration of cytokines (LCC: 12.5pg/ml IL-12 and 10ng/ml IL-18) **(A-B**), or inactivated whole H1N1 influenza virus (H1N1 with LCC) **(C-D)**. Responses were measured as the percentage of NK cells expressing CD25, CD25/IFN-γ, and CD107a, and compared between female (F) and male (M) donors for all donors **(A, C)**, or stratified into HCMV- (-) and HCMV+ (+) donors **(B, D)**. Data were analysed using two-tailed Mann-Whitney tests. * $p < 0.05$. Each data point represents one donor, n = 152 (24 experiments) **(A-B)**, n = 52 (18 experiments) **(C-D)**. Bars represent medians.

between HCMV- and HCMV+ donors, but all CD25+ responses, responses to vaccine with LCC, and responses to HCC, remain significantly lower in HCMV+ compared to HCMV- donors.

Finally, no associations were observed between anti-HCMV IgG titre and any NK cell responses among the HCMV+ subjects (data not shown), and there was no consistent effect of *NKG2C* genotype (which may affect NK cell differentiation [28,42,43]) on NK cell responses (Figure 27). For example, while there was a gene dosage effect between the number of copies of *NKG2C* and the percentage of NKG2C+ cells (Figure 27C, D), the heterozygous *NKG2C* $^{+/-}$ genotype was only statistically significantly associated with lower responses as compared to the homozygous *NKG2C* $^{+/+}$ genotype for some read-outs in HCMV- donors (Figure 27E). Although there were insufficient donors with the *NKG2C* $^{-/-}$ genotype to include in the comparisons, the allele frequency was comparable here between HCMV- and HCMV+ groups (Table IV) and thus I do not anticipate genotype to have a significant confounding impact on the analyses presented here. The effect of *NKG2C* genotype on the functional differentiation of NK cells in a population with near 100% HCMV seroprevalence is the subject of another publication by our group (Appendix VIII; [28]).

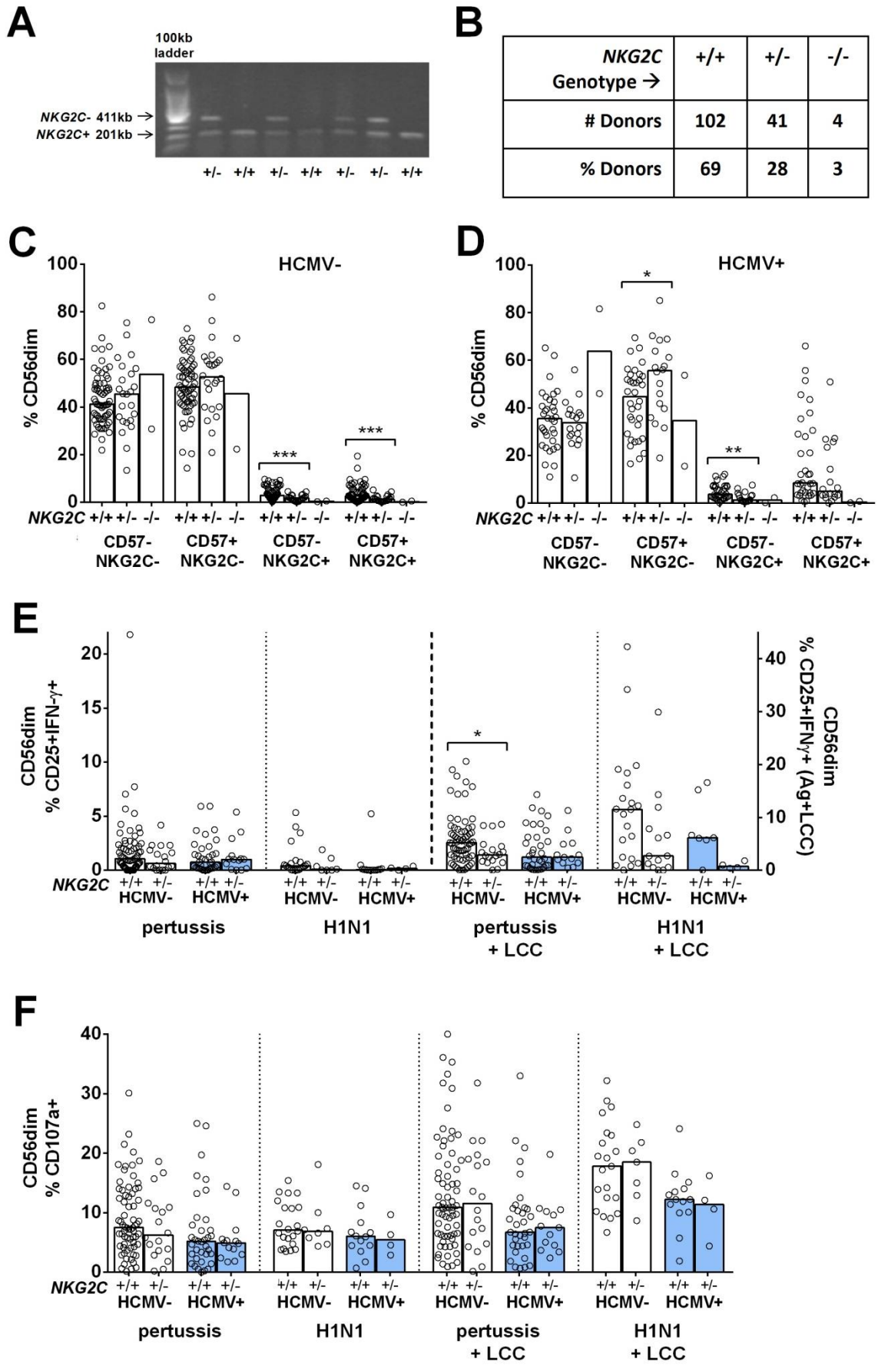

Figure 27. Stratification of NK cell responses by *NKG2C* genotype and potential gene dosage effect. Donors were genotyped for *NKG2C* using a nested PCR, as described in Methods. Gel electrophoresis was used to detect wild type *NKG2C* (201 kb **… (continued on page 127)**

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… [kilo-base pair] fragment) and *NKG2C* deletions (411 kb fragment), and thus determine the genotype of donors as *NKG2C*$^{-/-}$, *NKG2C*$^{-/+}$, or *NKG2C*$^{+/+}$ **(A-B)**. PBMC were analysed *ex vivo* for surface expression of CD57/NKG2C (see Figure 25 for gating strategy), and proportions of CD56dim NK cells in each of the four subsets were compared between *NKG2C*$^{-/-}$, *NKG2C*$^{-/+}$ and *NKG2C*$^{+/+}$ individuals in HCMV- donors **(C)** and HCMV+ donors **(D)**. Due to the small sample size of the *NKG2C*$^{-/-}$ group, statistical comparisons were restricted to the *NKG2C*$^{-/+}$ and *NKG2C*$^{+/+}$ groups. CD56dim *in vitro* CD25/IFN-γ **(E)** and CD107a **(F)** responses in *NKG2C*$^{-/+}$ and *NKG2C*$^{+/+}$ individuals to killed whole cell pertussis or inactivated whole virus H1N1 were also compared -/+ a low concentration of cytokines (LCC: 12.5pg/ml IL-12 and 10ng/ml IL-18), stratifying by HCMV serostatus. Note that CD25/IFN-γ responses to pertussis or H1N1 with LCC (Ag+LCC) are plotted on the right-hand axis **(E)**. Statistical analyses were performed using two-tailed Mann-Whitney tests. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Each data point represents one donor, n = 147 **(C-D)** or n = 143 **(E-F)**, and bar graphs denote medians. Genotyping data are from 5 different PCR experiments. *Ex vivo* and *in vitro* data are each from 24 experiments.

4.3.4 NK cell differentiation only partially explains reduced responses to vaccines in HCMV+ donors

I hypothesised that reduced cytokine-mediated NK cell responses among HCMV+ donors would reflect expansion of the highly differentiated CD56dimCD57+NKG2C+ NK cell subset which is known to be hyporesponsive to cytokines [6]. Indeed, *ex vivo* analysis confirmed observations from previous studies that HCMV+ donors had lower proportions of CD56dimCD57- NK cells and higher proportions of CD56dimCD57+ NK cells than did HCMV- donors (Figure 28A-B); there was no difference between the groups in the proportion of cells with intermediate CD57 expression (CD56dimCD57int, gating shown in Figure 28A). Consistent with previous work [4-6,10,11], HCMV seropositivity was also associated with a higher proportion of CD16+ (Figure 28C) and NKG2C+ (Figure 28D) cells, and a lower proportion of NKG2A+ cells (Figure 28E), within the total NK cell population. Moreover, HCMV seropositivity was correlated with a lower proportion of CD57-NKG2C- cells and a higher proportion of CD57+NKG2C+ cells within the CD56dim NK cell population (Figure 28F). HCMV+ donors also had lower expression of CD161 (associated with high NK cell IFN-γ production [44]) and higher expression of ILT2 (an inhibitory receptor, also known as LIR1, LILRB1, CD85j) on NK cells (Figure 29). This HCMV association with ILT2, though not significant with a two-tailed test, is in

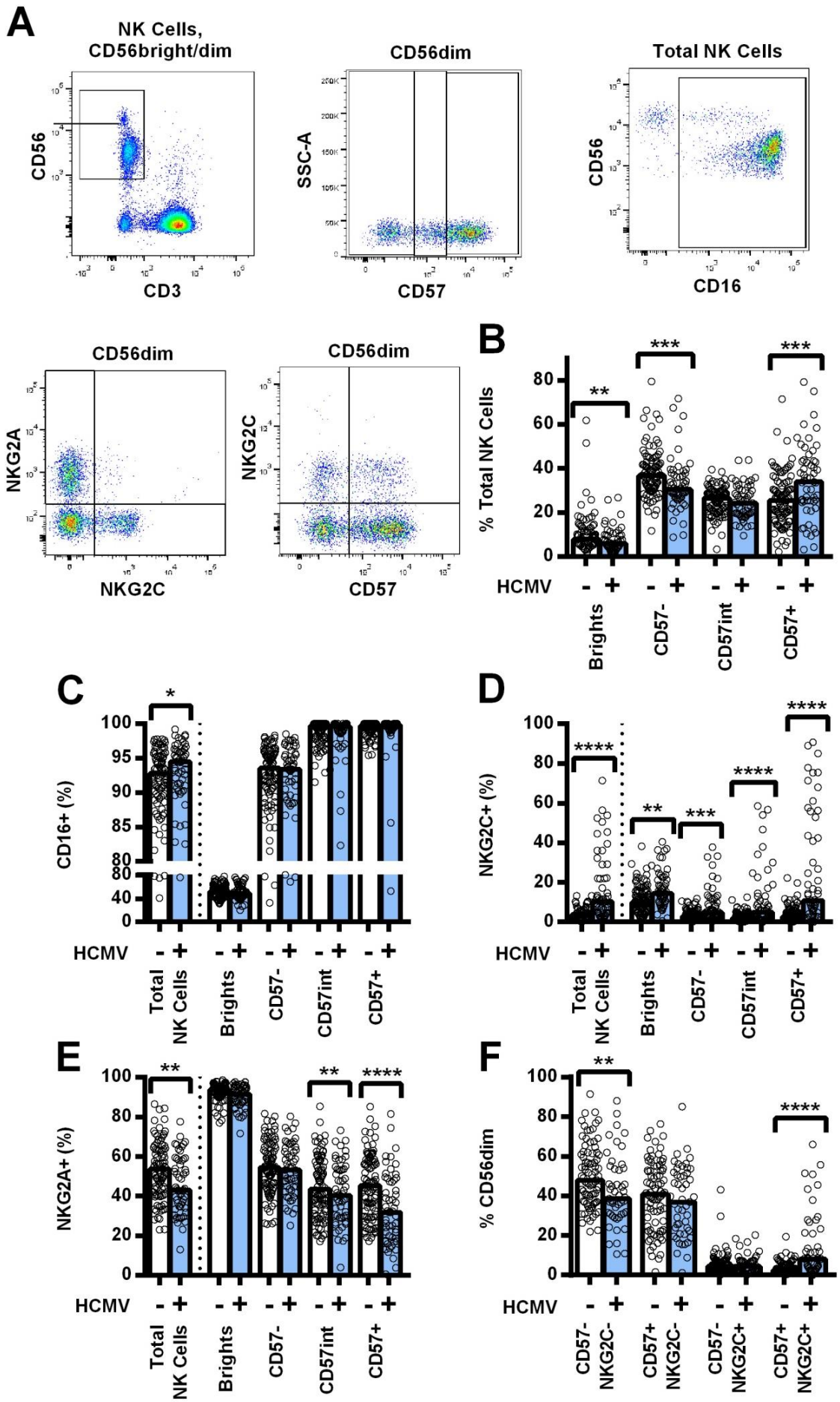

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Figure 28. Comparison of *ex vivo* expression of NK cell markers and receptors in HCMV- and HCMV+ donors. PBMC were analysed *ex vivo* for surface expression of CD56, CD57, CD16, NKG2C, and NKG2A, as shown by representative flow cytometry plots **(A)**. Proportions of total NK cells in the CD56bright, CD56dimCD57-, CD56dimCD57int, and CD56dimCD57+ subsets were compared between HCMV-and HCMV+ donors **(B)**, as was expression of CD16 **(C)**, NKG2C **(D)**, NKG2A **(E)**, and CD57/NKG2C (**F**, CD56dim only) The percentages of cells expressing each marker in HCMV- (-) and HCMV+ (+) donors were compared using two-tailed Mann-Whitney tests. **** $p \leq 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Each data point represents one donor, $n = 152$ (24 experiments), and bar graphs denote medians.

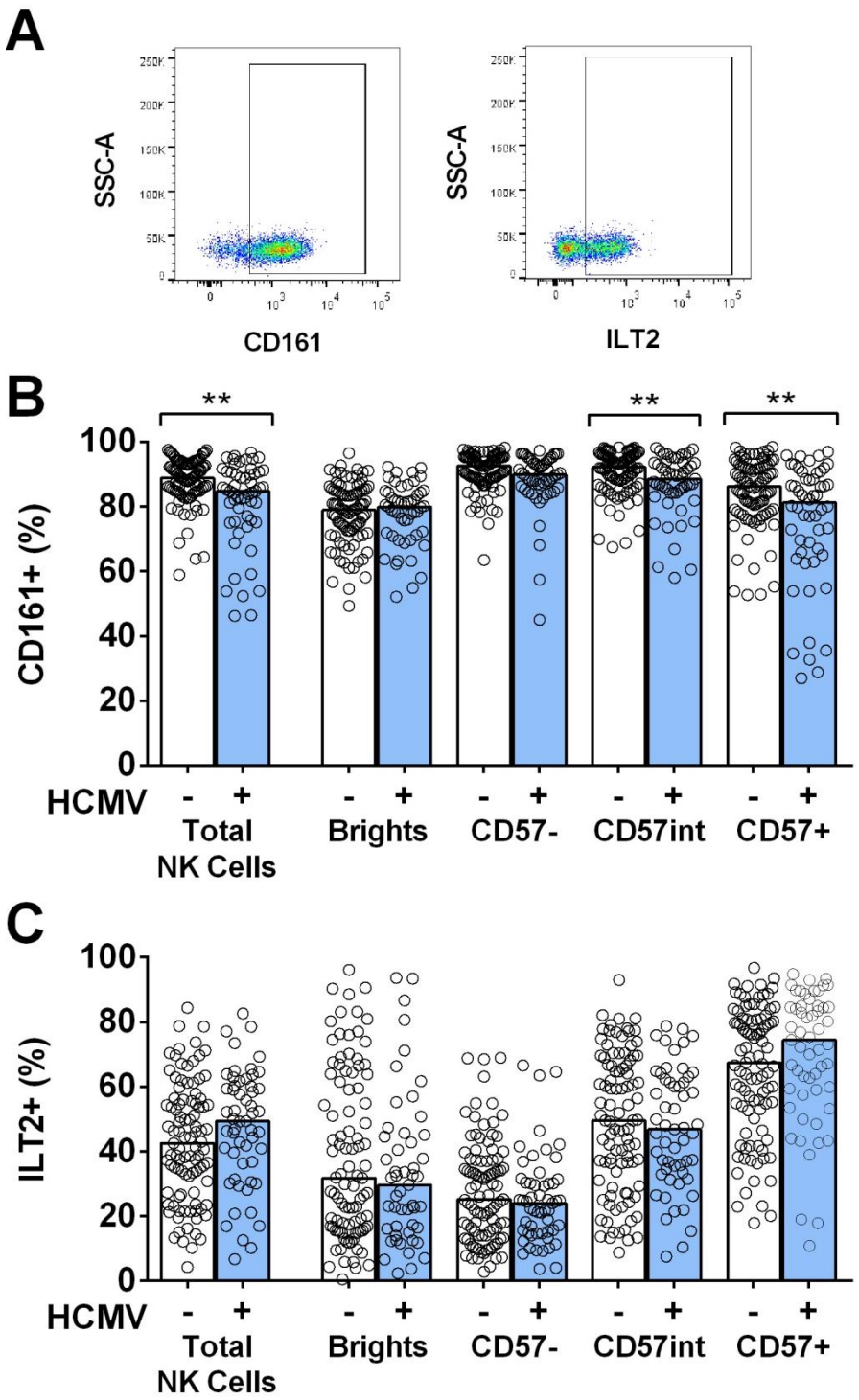

Figure 29. Comparison of *ex vivo* expression of CD161 and ILT2 on HCMV- and HCMV+ donors. PBMC were analysed *ex vivo* for surface expression of CD56, CD57, CD161 and ILT2. Gating of CD161 and ILT2 on total NK cells is shown by representative flow cytometry plots **(A)**. Proportions of total NK cells, and NK cells in the CD56bright, CD56dimCD57-, CD56dimCD57int, and CD56dimCD57+ subsets expressing CD161 **(B)**, and ILT2 **(C)** were compared between HCMV- (-) and HCMV+ (+) donors were compared using two-tailed Mann-Whitney tests. **** $p \leq 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Each data point represents one donor, $n = 152$ (24 experiments), and bar graphs denote medians.

agreement with previous publications, whereas published data is more inconsistent regarding the relationship between HCMV serostatus and CD161 expression [4,42,45-48]. No correlations were observed between ILT2 or CD161 and any functional read-outs (data not shown).

Whilst the increased proportion of CD56dimCD57+ NK cells among HCMV+ donors likely contributes to their reduced responsiveness to cytokines, I also observed significantly reduced CD25, CD25/IFN-γ, and CD107a expression in response to both pertussis and H1N1 *within* individual NK cell subsets. This was especially evident among CD56dimCD57+ cells and for cultures containing LCC (Figure 30A-F), but was also the case for cultures stimulated with vaccine alone (Figure 31G-I, M-O). Similarly, when cells were grouped by expression of CD57 and NKG2C, I found that responses to pertussis with LCC were lower among NKG2C+ NK cells than among NKG2C- cells (Figure 30G-I). This association was statistically significant for CD57+ NK cells of HCMV+ donors, but evaluation of the HCMV- cohort lacked statistical power as too few donors had sufficient NKG2C+ cells to allow a robust analysis. Interestingly, however, responses of all four subsets were significantly lower among HCMV+ donors than among HCMV- donors (Figure 30G-I), despite minimal differences in responses to LCC alone (Figure 31A-F). These data indicate that the reduced response of HCMV+ donors reflects differences in the intrinsic responsiveness of NK cells within a subset as well as differences in the distribution of these subsets. Although the level of expression (MFI) of both CD57 and NKG2C was higher on CD56dimCD57+ NK cells in HCMV+ donors compared to HCMV- donors (median MFI CD57 8901 vs 7245, $p = 0.0008$; median MFI NKG2C 137 vs 73, $p < 0.0001$), there was no significant association between CD57 and NKG2C expression levels and NK cell responsiveness in HCMV+ donors (Figure 32).

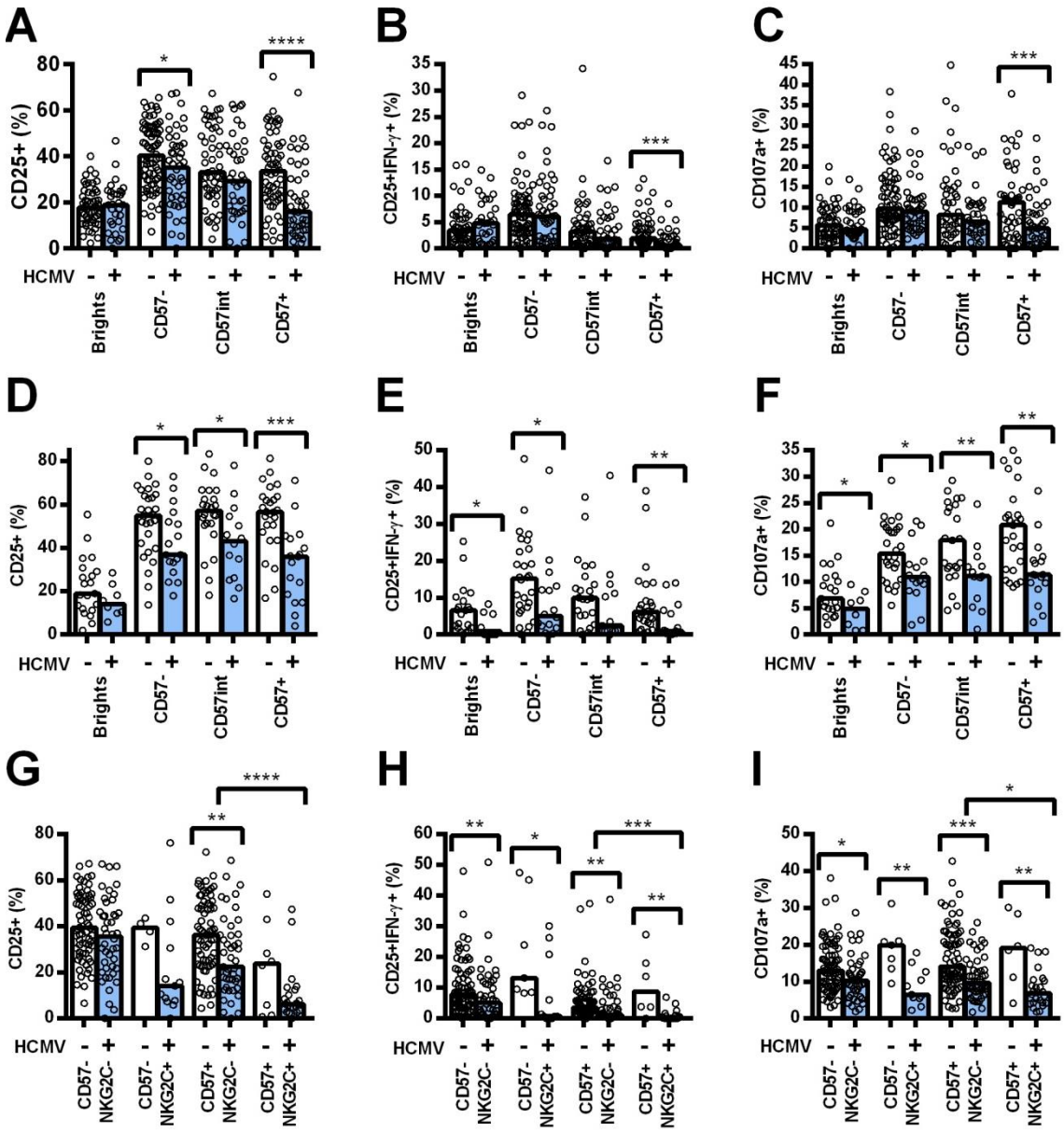

Figure 30. HCMV infection affects vaccine antigen responses of all NK cells, irrespective of their differentiation status. PBMC were cultured *in vitro* for 18 hours with killed whole cell pertussis with a low concentration of cytokines [(LCC: 12.5pg/ml IL-12 and 10ng/ml IL-18), pertussis + LCC] **(A-C, G-I)** or inactivated whole H1N1 influenza virus (H1N1 + LCC) **(D-F)**. Responses were measured as the percentage of cells expressing CD25 **(A, D, G)**, CD25/IFN-γ **(B, E, H)**, and CD107a **(C, F, I)** by CD56/CD57-defined subsets **(A-F)**, or CD56dim CD57/NKG2C-defined subsets **(G-I)** and compared between HCMV- (-) and HCMV+ (+) donors. Data were analysed using one-tailed Mann-Whitney tests. **** $p \leq 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Each data point represents one donor, $n = 152$ (24 experiments) **(A-C, G-I)**, or $n = 52$ (18 experiments) **(D-F)**, and bar graphs denote medians.

NB, for CD57/NKG2C-defined subsets, CD57int cells were grouped together with CD57- cells.

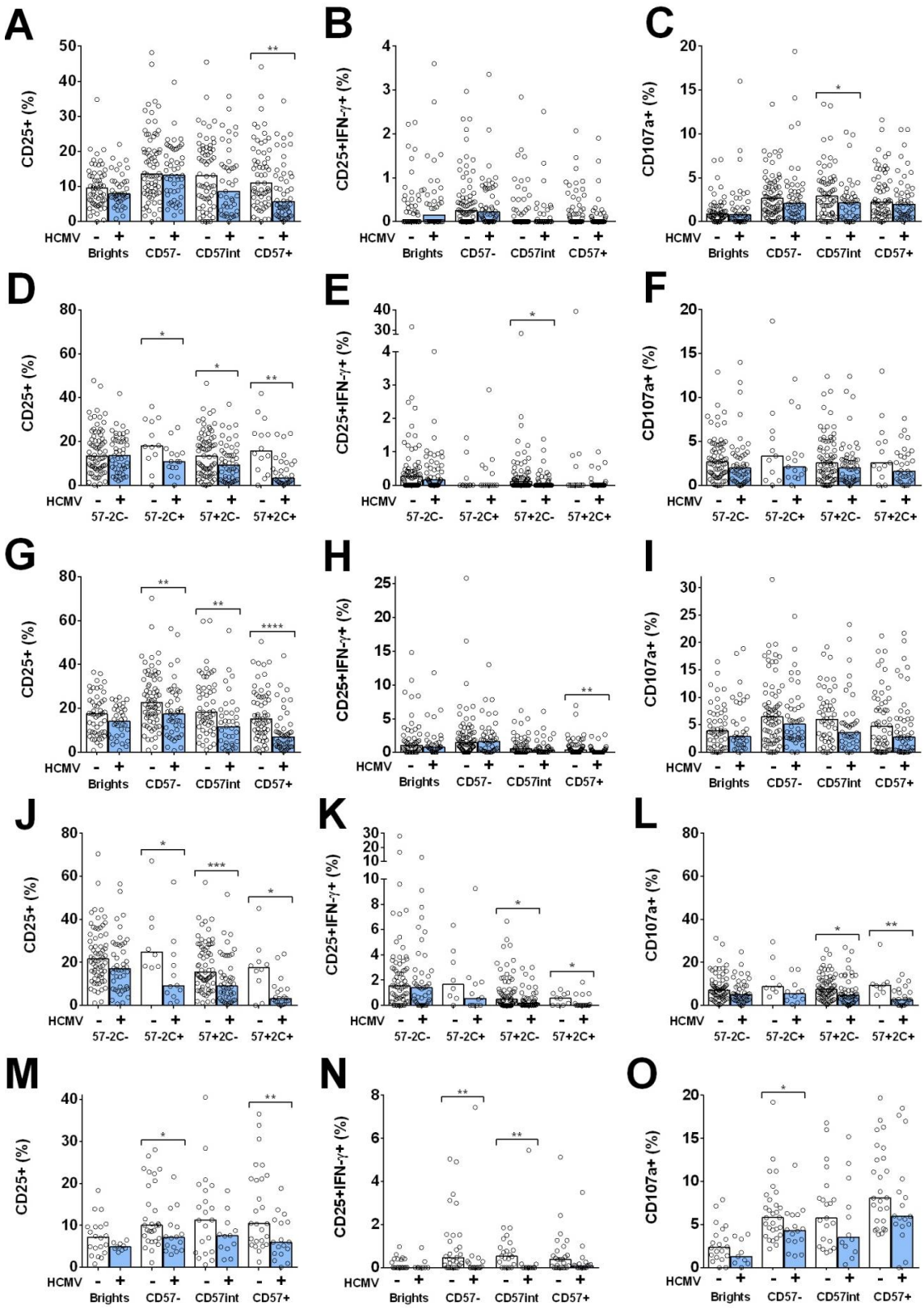

Figure 31. NK cell subset responses to LCC or vaccine antigen alone by HCMV status. PBMC were cultured *in vitro* for 18 hours with a low concentration of cytokines (LCC: 12.5pg/ml IL-12 and 10ng/ml IL-18)**(A-F)**, killed whole cell pertussis **(G-L)** or inactivated whole H1N1 influenza virus **(M-O)**. Responses were measured as the percentage of CD57-defined **(A-C, G-I, M-O)** or CD57/NKG2C-defined **(D-F, J-L)** NK cells expressing CD25 **(A, D, G, J, M)**, CD25/IFN-γ **(B, E, H, K, N)**, and CD107a **(C, F, I, L, O)** and were compared between HCMV- (-) and HCMV+ (+) donors. **… (continued on page 134)**

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… Data were analysed using one-tailed Mann-Whitney tests. **** $p \leq 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Each data point represents one donor, $n = 152$ (24 experiments) **(A-L)** or $n = 52$ (18 experiments) **(M-O)**, and bar graphs denote medians.

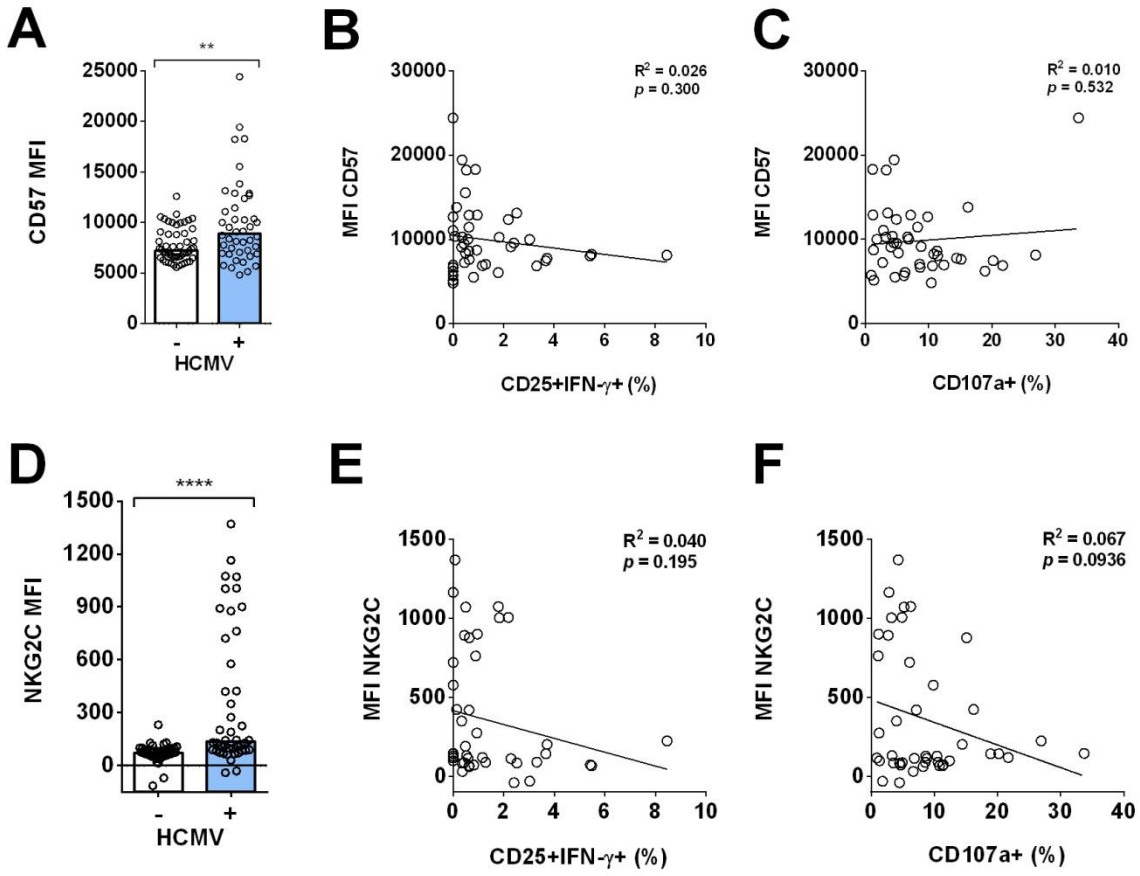
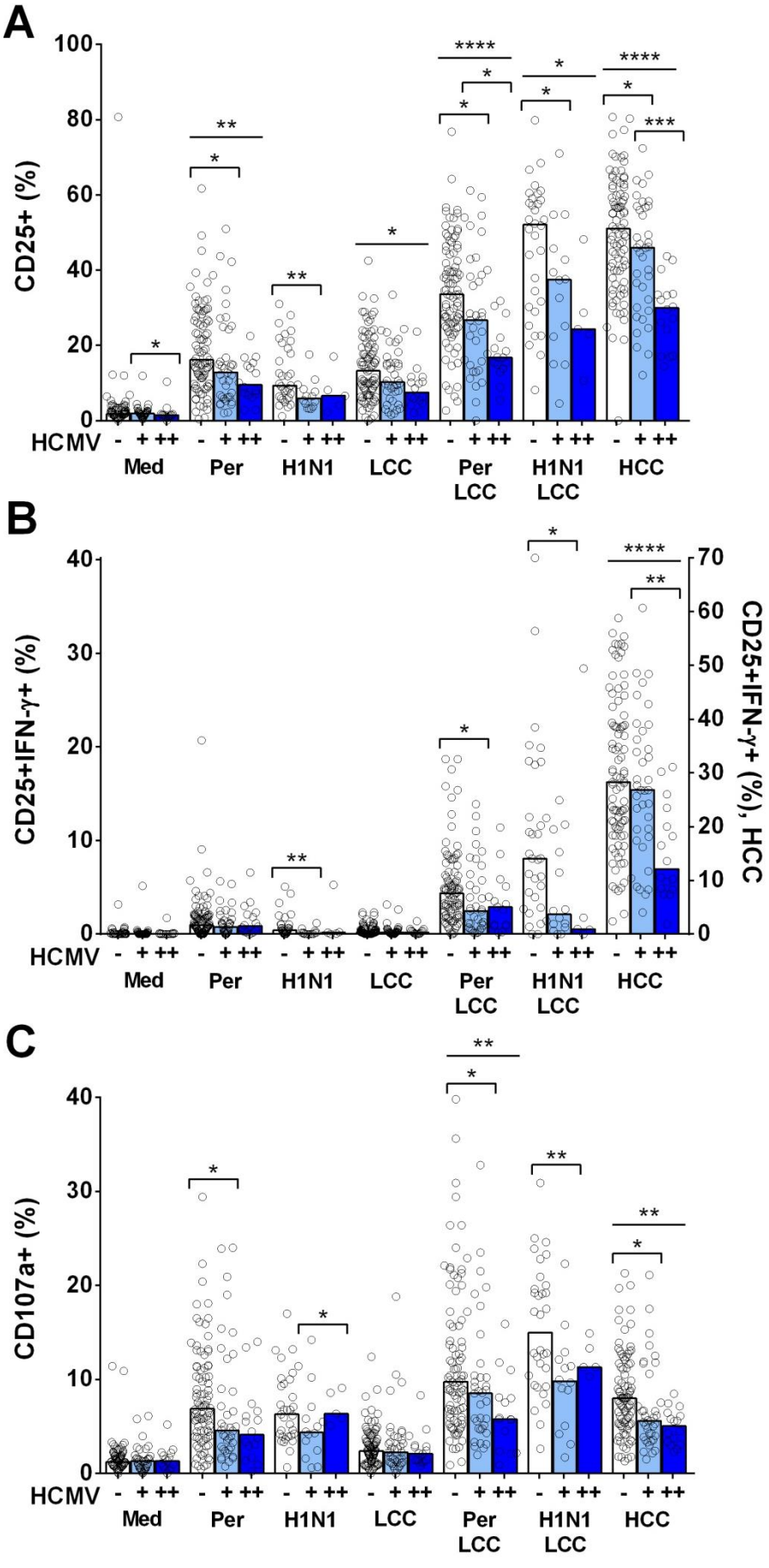


Figure 32. Higher expression of CD57 and NKG2C on CD56dimCD57+ subset in HCMV+ donors does not correlate with reduced NK cell responsiveness to pertussis. PBMC were cultured *in vitro* for 18 hours with killed whole cell pertussis and a low concentration of cytokines (12.5pg/ml IL12, 10ng/ml IL-18; LCC). CD56dimCD57+ NK cell expression of CD57 was measured as the geometric mean fluorescence intensity (MFI; **A**) and compared between HCMV- (-) and HCMV+ (+) donors. CD56dimCD57+ MFI of CD57 was correlated against CD56dimCD57+ responses to pertussis with LCC in terms of co-expression of CD25/IFN-γ **(B)** or CD107a **(C)**. CD56dimCD57+ NK cell expression of NKG2C was also measured as MFI **(D)** and compared between HCMV- and HCMV+ donors (D), then correlated against CD56dimCD57+ CD25/IFN-γ **(E)** or CD107a **(F)** responses to pertussis with LCC. Comparisons between HCMV- and HCMV+ donors were done using unpaired, two-tailed Mann-Whitney tests and correlations analyses with all donors were performed using bivariate regression. *** $p < 0.001$. Each data point represents one donor, $n = 152$ (24 experiments). Bar graphs denote medians.

Since only some HCMV+ individuals have obvious expansion of the CD56dimCD57+NKG2C+ subset I considered whether NK responses might differ between HCMV+ individuals with and without this expanded population. Sixteen of 55 (29%) HCMV+ donors demonstrated expansion of the CD56dimCD57+NKG2C+ subset (defined here as % CD56dimCD57+NKG2C+ cells greater than the mean + 3 standard deivations of that in HCMV- donors) and NK cells from these donors tended to respond less robustly than did cells from HCMV+ donors without this expansion (Figure 33). Importantly, there was evidence by trend analysis for decreasing NK cell responsiveness with HCMV infection, and then with HCMV infection plus expansion of the CD56dimCD57+NKG2C+ subset (Figure 33). This confirms that whilst expansion of the CD56dimCD57+NKG2C+ subset is associated with loss of NK cell responsiveness in vaccine recall assays, cells of HCMV+ donors respond less well than do cells of HCMV- donors, irrespective of NKG2C expression, i.e. even in the absence of the characteristic CD56dimCD57+NKG2C+ NK cell expansion..

4.3.5 HCMV+ donors retain capacity to respond to antibody-antigen complexes

Although there was a clear role for specific IgG in induction of CD25, CD25/IFN-γ, and CD107a expression (Figure 19), impairment of CD16-mediated signalling seemed an unlikely explanation for reduced NK cell responsiveness since HCMV+ individuals have a higher frequency of CD16+ NK cells (Figure 28C), cells from HCMV+ and HCMV- donors responded equally well to CD16 crosslinking (Figure 34B) — demonstrating no intrinsic difference in the ability of NK cells from HCMV- and HCMV+ donors to degranulate — and, use of pooled AB plasma for *in vitro* assays ensured that specific IgG concentrations were consistent in all assays. Indeed, using pre-incubation with IL-2 to improve ability to detect responses to crosslinking (Figure 34A), I demonstrated that cells from HCMV- and HCMV+ subjects respond equally well to NKp30/NK46 crosslinking or K562 stimulation, as well as CD16 crosslinking (Figure 34B-D). This clearly indicates that HCMV+ donors' NK cells have the capacity to degranulate. Furthermore, there were no consistent differences in the degranulation



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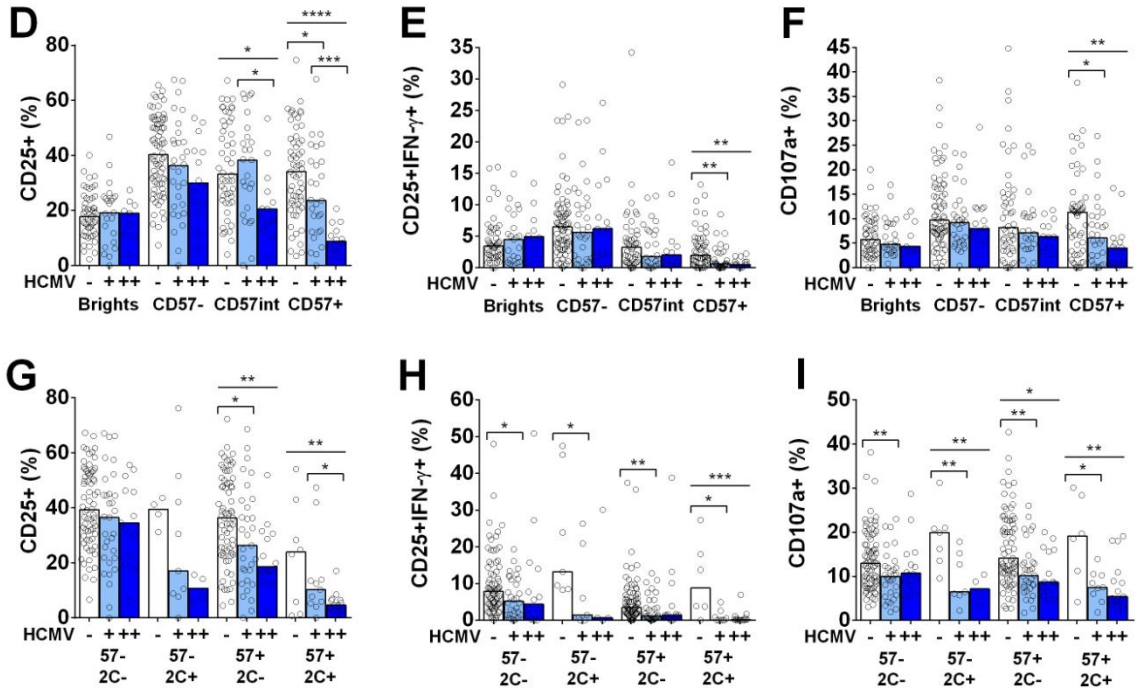

Figure 33. NK cell responses to vaccine antigen by HCMV+ donors without the characteristic CD56dimCD57+NKG2C+ expansion may reflect an intermediate phenotype between HCMV- and HCMV+ donors. PBMC were cultured *in vitro* for 18 hours with medium alone (Med), low concentration of cytokines (LCC: 12.5pg/ml IL-12 and 10ng/ml IL-18), killed whole cell pertussis (Per), inactivated whole H1N1 influenza virus (H1N1), Per + LCC, H1N1 + LCC, or high concentration of cytokines (HCC: 5ng/ml IL-12, 50ng/ml IL-18). Donors were stratified into HCMV- (-), HCMV+ without a CD56dimCD57+NKG2C+ expansion (+), and HCMV+ with a CD56dimCD57+NKG2C+ expansion (++). Responses were measured as the percentage of total NK cells expressing CD25 **(A)**, co-expressing CD25/IFN-γ **(B)**, or CD107a **(C)**. CD57-defined **(D-F)** or CD57/NKG2C-defined subsets **(G-I)** were analysed for responses to pertussis with LCC for CD25 **(D, G)**, CD25/IFN-γ **(E, H)**, and CD107a **(F, I)**. Data were analysed using, one-tailed Mann-Whitney tests to compare responses between HCMV+ donors and either HCMV- donors or HCMV++ donors. Analysis of variance for linear trend (from - to + to ++) was also performed for each functional readout (uncapped lines). **** $p \leq 0.0001$, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Each data point represents one donor, $n = 152$ (24 experiments), except for H1N1 and H1N1 + LCC where $n = 52$ (18 experiments). Bar graphs denote medians.

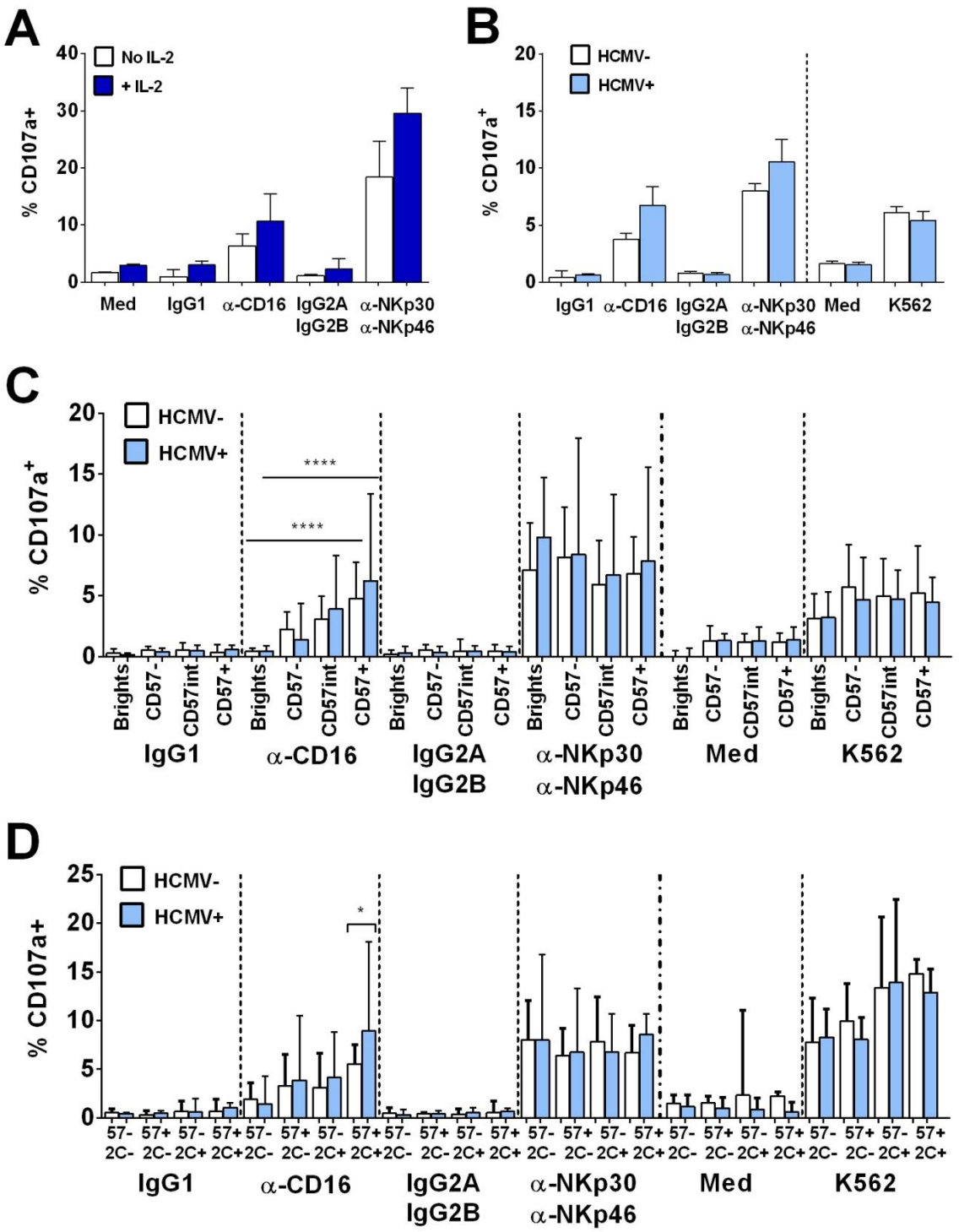

Figure 34. Degranulation responses after CD16, NKp30/NKp46 crosslinking or K562 stimulation are equally robust in HCMV- and HCMV+ donors. In preliminary experiments, PBMC were cultured *in vitro* for 5 hours in a 96-well flat-bottomed plate coated with medium alone (Med), IgG1 isotype control, anti-CD16, IgG2A/ IgG2B isotype controls, or anti-NKp30/NKp46 after overnight incubation with or without 100IU/ml IL-2. Total NK cell responses were measured the percentage expressing CD107a **(A)**. Subsequently, PBMC were cultured *in vitro* for 5 hours in a 96-well flat-bottomed plate coated with IgG1 isotype control, anti-CD16, IgG2A/ IgG2B isotype controls, or anti-NKp30/NKp46 after overnight incubation with 50IU/ml IL-2. PBMC were also cultured *in vitro* for 18 hours in a 96-well round-bottom plate with medium alone (Med) or K562 cells, at an **… (continued on page 139)**

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… effector:target (E:T) ratio of 2:1. Total NK cell CD107a responses were compared between HCMV- and HCMV+ donors **(A)**. Responses were then stratified based on CD57-defined subsets **(C)** or CD57/NKG2C-defined subsets **(D)**. Comparisons between HCMV- and HCMV+ were performed using two-tailed Mann-Whitney tests. ANOVA test for linear trend for responses to CD16 crosslinking with acquisition of CD57 was also performed for HCMV- and HCMV+ donors. . **** $p \leq 0.0001$* $p < 0.05$. $n = 4$, data is from a single experiment **(A)**, $n = 48$ (17 experiments) **(B-D)**, or $n = 100$ (6 experiments) (**B-D**, Med and K562). Note that activating receptor crosslinking and K562 experiments were performed with different donors. Bars represent medians and lines denotes interquartile ranges.

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responses across CD57- or CD57/NKG2C-defined subsets between HCMV- and HCMV+ donors (Figure 34C-D) and both groups showed a trend to increasing responsiveness to CD16 with acquisition of CD57 ($p < 0.0001$; Figure 34C). The exception to this is the superior CD16 crosslinking response of CD56dimCD57+NKG2C+ in HCMV+, as compared to the same subset in HCMV- donors, which is in line with published reports of enhanced responses of this subset to HCMV-infected target cells only in the context of HCMV-specific antibody [7,10,49].

4.3.6 HCMV infection is associated with altered expression of cytokine receptors by NK cells

On the other hand, differences between HCMV+ and HCMV- donors were most marked in cultures containing LCC (e.g. Figures 21, 30), and in cultures with high concentrations of the cytokines IL-12 and IL-18 (HCC; Figure 35A-C), suggesting that differences in expression of cytokine receptors might explain our observations. Although there was no difference in resting (*ex vivo*) expression of IL-12Rβ2 on any NK cell subset (Figure 35E), IL-12Rβ2 was significantly upregulated on the total NK cell population in HCMV- but not from HCMV+ donors after culture with HCC (Figure 35F). Moreover, and consistent with data showing associations between acquisition of CD57 and increased IL-18Rα expression [2,40,41], resting NK cells from HCMV+ donors were significantly less likely than cells from HCMV- donors to express IL-18Rα and this difference was especially marked in the (expanded) CD56dimCD57+ NK cell subset (Figure 3H). Nonetheless, *ex vivo* expression of IL-18Rα cannot be solely responsible for the

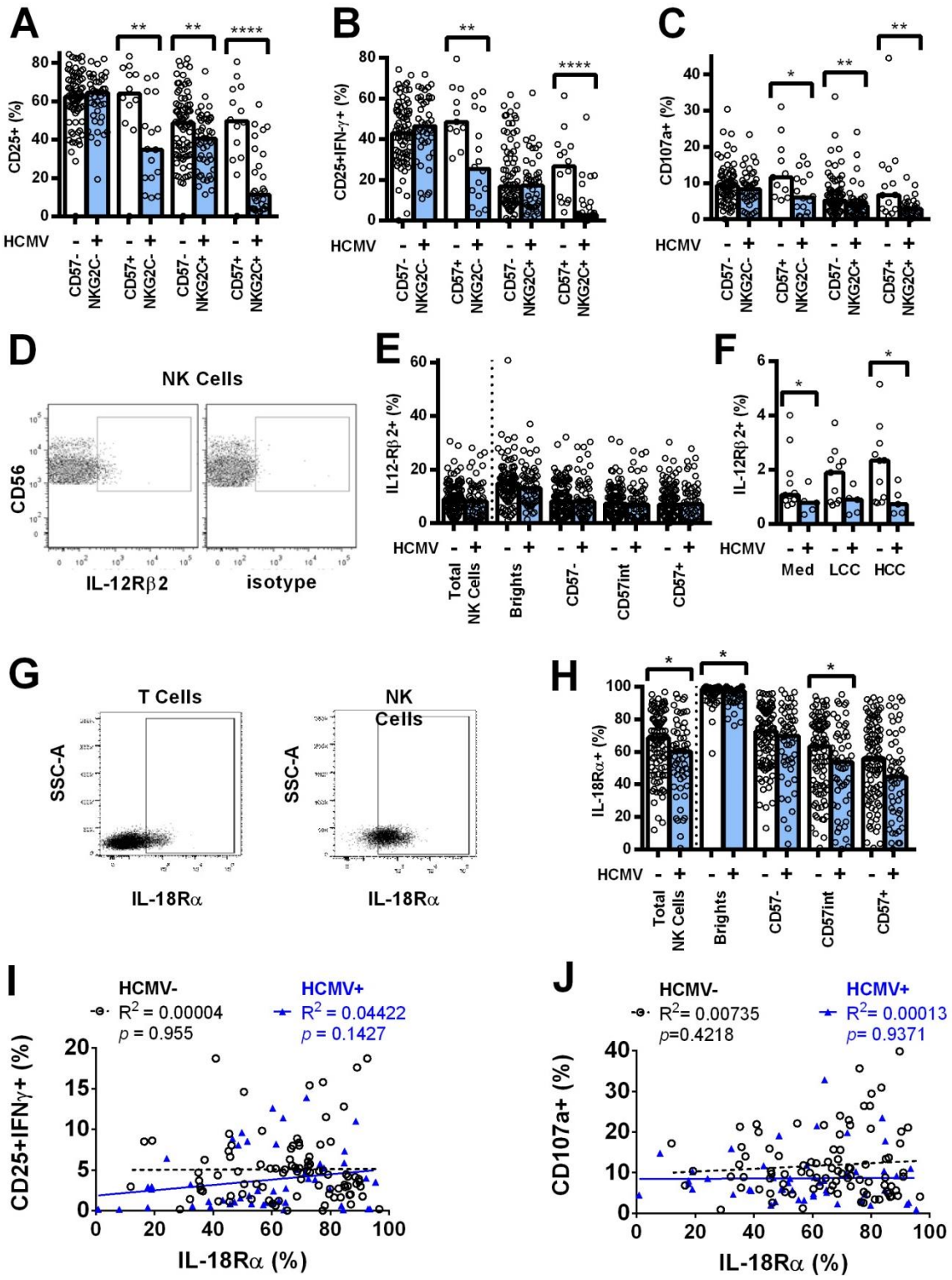

Figure 35. Decreased cytokine responsiveness and decreased cytokine receptor expression by NK cells from HCMV+ donors. PBMC were cultured *in vitro* for 18 hours with a high concentration of cytokines (HCC: 5ng/ml IL-12, 50ng/ml IL-18)**(A-C)**. Responses were measured as the percentage of CD56dim CD57/NKG2C-defined cells expressing CD25 **(A)**, CD25/IFN-γ **(B)**, and CD107a **(C)** and compared between HCMV- (-) and HCMV+ (+) donors **(D-F)**. NK cells were analysed for surface expression of IL-12Rβ2 using a mIgG1 PECy5-conjugated isotype control to set the gate, as performed by Martin Goodier **(D)**. Total NK cells **(E-F)** and CD56/CD57-defined subsets **(E)** were analysed *ex vivo* **(E)** and after 18 hours culture *in vitro* with low concentration of cytokines (LCC: 12.5pg/ml IL-12 and 10ng/ml IL-18) or HCC **(F)**… **(continued on page 141)**

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… NK cells were also analysed for IL-18Rα surface expression using the T cell population to set the IL-18Rα gate **(G)**, for total NK cells and CD56/CD57-defined subsets *ex vivo* **(H)**. Correlation between *ex vivo* expression of IL-18Rα by total NK cells and HCMV- or HCMV+ CD25/IFN-γ **(I)** or CD107a **(J)** responses to pertussis with LCC *in vitro* were analysed by bivariate regression. HCMV- and HCMV+ donors were compared using one-tailed **(A-C)** or two-tailed **(E-F, H)** Mann-Whitney tests. **** $p \leq 0.0001$, ** $p < 0.01$, * $p < 0.05$. Each point represents one donor, $n =$ 152 (24 experiments) **(A-C, E, H-J)**, or $n =$ 16 (one experiment, performed by Matt White and Scarlett Turner) **(F)**, and bar graphs denote medians.

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differences observed in vaccine responses between HCMV- and HCMV+ donors as no significant correlation between IL-18Rα and *in vitro* responses to pertussis with LCC were observed (Figure 35I, 35J).

4.4. Discussion

During secondary immune responses, both CD4+ T cell-derived IL-2 and antigen-antibody immune complexes induce 'antigen-specific' NK cell activation, allowing NK cells to act as effectors of the adaptive immune response and to contribute to post-vaccination immunity by secretion of IFN-γ and/or by cytotoxicity [2,8,9,32,50]. Here I demonstrate, for the first time, that the contribution of NK cells to adaptive immune responses is affected by HCMV infection: NK cells from HCMV+ donors respond significantly less well than cells from HCMV- donors to killed whole cell pertussis or inactivated whole H1N1 influenza virus. The effect of HCMV infection on NK cell responsiveness is independent of age, sex, or anti-HCMV IgG titre.

My data also demonstrate, for the first time, that there is an additive effect between the cytokine and the IgG pathways driving NK cell IFN-γ production, as both IgG depletion and IL-2 blockade reduced NK cell IFN-γ responses in response to stimulation of PBMCs with pertussis vaccine. Of particular interest, IgG depletion markedly reduced antigen-induced CD25 expression on NK cells. I propose that CD16 crosslinking by immune complexes upregulates CD25 expression, increasing sensitivity to T cell-derived IL-2 and thereby enhancing IFN-γ production. However, CD16 crosslinking is not essential for upregulation of CD25, as this can be induced by antigen alone, presumably in response to IL-12 and IL-18 produced by APCs [2,51-53]. Release of cytotoxic granules, as measured by upregulation of CD107a on the cell surface, is also inhibited by IgG depletion but is unaffected by IL-2 blockade, suggesting that NK cells could act as effectors of the adaptive response through ADCC in the absence of memory T cells, providing there was sufficient circulating antibody. These interactions are further explored in Chapter 5.

However, while IgG depletion also decreased H1N1-induced CD25 expression on NK cells, H1N1 induction of IFN-γ was significantly enhanced in the absence of IgG. I have observed that individual NK cells tend to either produce IFN-γ or degranulate (but not both; unpublished

data) suggesting that inhibiting the degranulation response to H1N1 by removing IgG skews the response towards IFN-γ production. However, given the limited effect of IgG depletion on H1N1-induced degranulation, it is unclear why this should be the case. Indeed, expression of CD107a in response to H1N1 seems to be relatively unaffected by either IL-2 blockade or IgG depletion. This suggests that H1N1-driven degranulation may be affected by other stimuli, such as type I interferons [54,55]. Since the start of this PhD work, Jegaskanda *et al* have also published on NK cell ADCC in response to influenza antigen-antibody complexes [56]. The authors commented that IgG depletion resulted in negligible NK cell degranulation or IFN-γ production in response to H3N2, but they did not include these IgG depletion results in the manuscript, precluding an evaluation alongside my data.

I had hypothesised that decreased responses to vaccines in HCMV+ donors would be attributable to a redistribution of the NK cell repertoire. HCMV infection drives the expansion of a CD56dimCD57+NKG2C+ subset of NK cells [5,10,11,57], which display a highly differentiated phenotype, including reduced responsiveness to exogenous cytokine stimulation [40,41]. These phenotypic and functional changes are similar to those observed during ageing [3,58] and comparisons have been drawn between the effects of HCMV and immunosenescence [59]. As our previous work has indicated that NK cell IFN-γ production after re-stimulation with vaccine antigens is cytokine-dependent [9], I predicted that fewer NK cells from HCMV+ donors would produce IFN-γ in response to pertussis or influenza antigens due to the reduced capacity of the expanded CD56dimCD57+NKG2C+ subset to respond to cytokines.

Ex vivo analyses confirmed that HCMV+ donors had higher proportions of CD56dimCD57+ and CD56dimCD57+NKG2C+ NK cells than did HCMV- donors and functional analysis confirmed that very few of the highly differentiated CD57+ NK cells produced IFN-γ after antigen stimulation. Interestingly, however, my data also show that — irrespective of their

CD57/NKG2C surface phenotype — NK cells from HCMV+ are less likely to produce IFN-γ in response to vaccine antigens than are cells from HCMV- donors. In other words, there are pronounced functional differences between HCMV+ and HCMV- donors *within* NK cell subsets. The reduced NK cell IFN-γ response to vaccine antigens in HCMV+ donors is therefore not simply due to expansion of the CD56dimCD57+NKG2C+ subset. Although acquisition of NKG2C was functionally relevant (associated with reduced IFN-γ and degranulation responses), it was not sufficient to explain the reduced responsiveness of cells from HCMV+ donors.

Whilst further studies are required to define the 'within subset' effects of HCMV infection, our data suggest that reduced expression of IL-18Rα or reduced ability to upregulate IL-12Rβ2 among NK cells from HCMV-infected individuals may partially explain their failure to produce IFN-γ. Although decreasing expression of IL-12Rβ2 and IL-18Rα expression have been associated with CD57 expression, this is the first demonstration that there are differences in cytokine receptor expression between HCMV+ and HCMV- donors and it is possible to see how each of these might affect NK cell responses. Higher resting levels of IL-18Rα expression would increase the sensitivity of NK cells to low concentrations of IL-18 being produced by APCs in response to innate receptor ligands in whole cell pertussis or inactivated influenza virus. IL-18 signalling upregulates CD25 [60] thereby increasing sensitivity to IL-2. IL-2 signalling might then upregulate IL-12R2β [61,62] allowing IL-12 to synergise with IL-2 to drive IFN-γ production [9,51,63], whilst also generating a positive feedback loop in which IL-12 signalling upregulates IL-18Rα [64,65], IL-18 signalling and CD25. These hypothesised feedback loops and synergies are explored in more detail in Chapter 5.

However, while cytokine receptor expression is likely to play a role in determining NK cell responsiveness to vaccine antigens in HCMV- and HCMV+ donors, the biological relevance of small changes in surface expression on IL-12Rβ2 needs to be demonstrated. Moreover, while I have no evidence to suggest that T cell IL-2 production in response to vaccine antigens is

affected by HCMV infection, future studies will need to determine the extent to which concomitant changes in APC function during HCMV infection also affect NK cell responses.

I had initially considered NK cell degranulation during vaccine re-stimulation to be a result of CD16 crosslinking by IgG immune complexes, as suggested by the IgG depletion data and the assumption that soluble signals broadly drive cytokine production rather than cytotoxicity (as illustrated in [66]). The expectation was, therefore, that although IFN-γ responses might be impaired, NK cell degranulation responses would be sustained in HCMV+ donors. Indeed, crosslinking with anti-CD16 antibody induced equivalent levels of CD107a upregulation. It was, therefore, somewhat surprising that degranulation responses to vaccine were lower in HCMV+ donors than in HCMV- donors. However, degranulation responses to HCC were also lower in HCMV+ donors, supporting the notion of synergy between the cytokine and CD16 pathways and adding weight to the suggestion that HCMV infection may affect cytokine receptor expression. This was an area of particular interest and is the focus of Chapter 5.

My findings have potentially important implications. HCMV infection is a known risk factor for all-cause mortality in adults [67] and perinatal HCMV infection is associated with slower growth and increased rates of hospitalisation in African children [68]. The underlying biology of these relationships is unknown but reduced responsiveness to vaccination or reduced resilience in the face of infection are plausible explanations. Distorted T cell and NK cell phenotypes in HCMV+ individuals have been widely reported (see Chapter 1 [3,69-71]) giving credence to the possibility that adaptive immune responses may be less effective in infected individuals. Further work will need to address the clinical consequences of altered NK cell responses to infection and vaccination in HCMV-infected individuals.

To my knowledge, this is the first published study of the effect of HCMV infection on NK cell responses to vaccine antigens. Our group have previously shown in an African population that,

with near universal infant HCMV infection, the characteristic 'adult HCMV' NK cell profile is reached by late childhood [28], which raises the intriguing question as to whether the duration of HCMV infection affects vaccine responses. The majority of the donors in this study are of European or North American origin suggesting that they may have been infected in adolescence or adulthood [72,73], potentially explaining some of the heterogeneity in the responses I see within the HCMV+ group. This uncertainty regarding age at infection and time since infection is in contrast to HCMV epidemiology in developing countries in sub-Saharan Africa, where seroprevalence reaches may near 100% in early childhood [28]. Indeed, there are clear differences between HCMV+ donors from my study in the UK, and age-matched HCMV+ donors in our study in the Gambia (Figure 36). Although there are certainly many other confounding variables between HCMV+ adults in the UK and the Gambia, average number of years since primary HCMV infection will clearly be one of these. Future studies will need to assess whether the duration of HCMV infection is a risk factor for altered NK responses and whether this manifests itself as reduced responsiveness to vaccination, recall responses to vaccine antigens, and reduced vaccine efficacy.

Similarly, there will also be variation among my donors in time since vaccination (pertussis) or infection (H1N1), and it is likely that the relatively low IFN-γ responses I observe (in comparison to earlier studies [9]) is due to the much longer interval between primary and secondary exposures to antigen. Longitudinal studies controlling for HCMV serostatus in vaccine intervention studies will provide insight into the impact of HCMV infection at the time of vaccination; an influenza vaccine intervention study conducted with 52 donors from this cohort indeed found a profound impact of HCMV on post-vaccination NK cell functionality over many months (Goodier, Lusa, Rodríguez-Galán, Nielsen *et al*, manuscript accepted). To note, it is possible that a small minority of donors had not been previously naturally exposed to H1N1, or that some of donors really had not received pertussis vaccination in childhood. Such

differences in true primary exposure would also contribute to the variation we observe in the NK vaccine response data.

Finally, it is of particular interest that HCMV+ donors without an expanded CD56dimCD57+NKG2C+ subset appear to represent an intermediate phenotype between HCMV- subjects and HCMV+ subjects with expanded NKG2C+ subset of NK cells ('HCMV++' donors). The cause of these differences between HCMV+ and HCMV++ donors are unclear, but may reflect time since HCMV infection, frequency of HCMV reactivation events, the genotype of HCMV, and host genetics. Clearly individuals with the *NKG2C* $^{-/-}$ genotype will be unable to expand a CD56dimCD57+NKG2C+ subset, and thus *NKG2C* genotype may will be another confounding factor contributing to donor heterogeneity. The very small numbers of *NKG2C* $^{-/-}$ donors in this study homologous precluded further investigation in this cohort, but the role of *NKG2C* deletions in NK cell functional biology continues to be of particular interest and a focus of work led by others in our group (Appendix VIII, [28]).

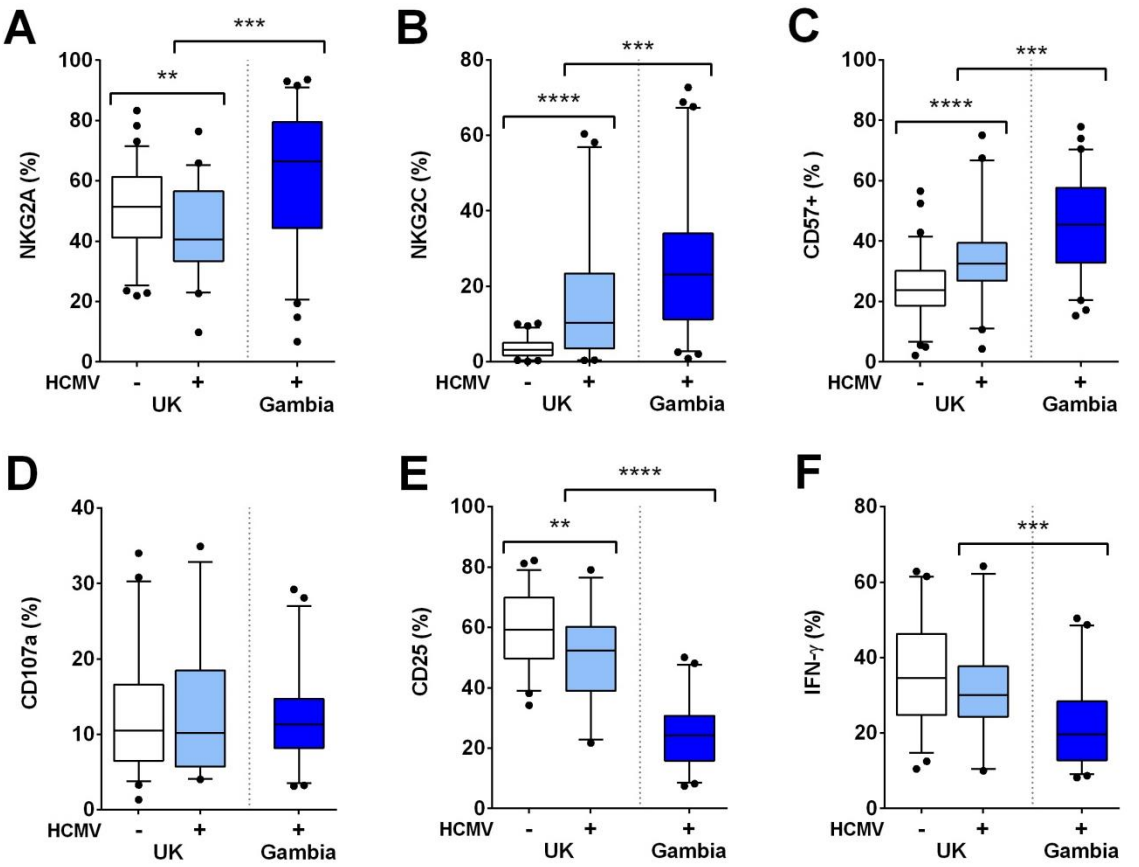

Figure 36. Elevated frequencies of NKG2C+ NK cell subsets and reduced NK cell functional capacity in HCMV+ Gambian compared to HCMV+ UK adults. The frequencies of NKG2A+ **(A)**, NKG2C+ **(B)** and CD57+ **(C)** within CD56dim NK cells are compared Gambian adults (*n* = 65) and HCMV- (*n*= 78) and HCMV+ UK adults (*n* = 43) aged between 20-49 years. Frequencies of cells expressing CD107a **(D)**, CD25 **(E)** and IFN-γ **(F)** were determined within total NK cells following stimulation with a high concentration of cytokines (HCC: 5ng/ml IL-12, 50ng/ml IL-18). ** $p <$ 0.0001. The central lines represent the medians, while the upper and lower limits of the box indicate the 25th and 75th percentiles. The whiskers denote the 5 and 95th percentiles while points are individual donors who are outliers outside the 5th and 95th percentiles. Adapted from [28].

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Chapter 5

Synergy between common γ chain family cytokines and IL-18 potentiates innate and adaptive pathways of NK cell activation

The work presented in thus chapter is adapted and extended from:

Nielsen*, Wolf*, Goodier, Riley. Synergy between common gamma chain family cytokines and IL-18 potentiates innate and adaptive pathways of NK cell activation. *Manuscript accepted*.

5.1. Introduction

The data presented in Chapters 3 and 4 indicate a central role for NK cell sensitivity to exogenous cytokines, as influenced by CD57-defined maturation status [1] and HCMV infection [2], in determining NK cell 'recall responses' to vaccine antigens [1,2]. Given the importance of pro-inflammatory cytokines IL-12 and IL-18 in activating NK cells during recall responses [3], and the decreased expression of IL-18Rα in HCMV+ individuals across all CD57-defined subsets [2], it seems plasuible that defects in cytokine receptor expression contribute to impaired vaccine responses in HCMV+ individuals. I have therefore sought to characterise the effects of pro-inflammatory cytokines, alone or in combination, on NK cell function in order to understand how this guides the key NK cell responses in the first hours following primary or secondary exposure to pathogens. Specifically, I was particularly interested to understand synergies involving IL-18 signalling, which could indicate whether the small differences in IL-18Rα expression detected in HCMV+ individuals are functionally relevant.

Additionally, while IL-12Rβ2 expression *ex vivo* was not significantly different between HCMV- and HCMV+ individuals, it is possible that cytokine receptor upregulation following activation during culture with either H1N1 or *Bordetella pertussis* did vary between HCMV- and HCMV+ groups (Chapter 4, [2]). The IL-12R has two chains, of which IL-12Rβ2 is the inducible component. I hypothesised therefore that NK cells from HCMV- donors are more sensitive to IL-2 signalling and, as IL-2 is reported to drive upregulation of IL-12Rβ2 [4,5], that IL-12Rβ2 kinetics may differ with HCMV serostatus. Similar relationships have been published between IL-18 and either IL-12 or IL-15, with the latter cytokines driving IL-18Rα upregulation [6,7].

However, while functional synergies between IL-2, IL-12, IL-15 and IL-18 are very likely partially attributable to feedback mechanisms on cytokine receptor expression at the NK cell surface *in vitro*, previously published data do not provide convincing evidence that this occurs in a physiological context. Most studies that have investigated these interactions use very high

concentrations of cytokines that would be unattainable *in vivo*, and/ or use isolated NK cells [6,7]. While purified cell populations have advantages, it is difficult to achieve 100% purity. Any contaminating T cells are likely highly activated due to the use of anti-CD3 beads in the NK isolation method, and may be producing cytokines that could affect the assay. Additionally, many studies use relatively long time courses, e.g. 24 hours [6,8], 72 hours [7], or 3 days [5]. The data from these experiments may reflect real changes in the NK cell population, but cytokine receptor expression at these later time points is unlikely to explain differences in responses after only 18 hours (i.e. as described in Chapter 4, [2]). More importantly perhaps, by these later time points the NK cell response will no longer dominate due to the increased contribution from T cells.

We chose to focus primarily on IL-12, IL-15, and IL-18, based on their key roles in pro-inflammatory responses and common use, by our lab and others, in NK cell stimulation assays. These cytokines vary in their function and cellular source. IL-15 is widely produced, including by dendritic cells, and is known for its role in NK and T cell homeostasis and maturation; it is an established survival signal in the bone marrow and is required for long-term culture of NK cells [9,10]. IL-18 is generated by macrophages and, alongside IL-12, can effectively activate both NK and T cells. IL-12 is thought to be secreted primarily by monocytes and be essential for IFN-γ production by NK and T cells (as reviewed in [11,12]).

Interestingly, IL-12 alone cannot drive significant IFN-γ production but robustly induces an IFN-γ response in the presence of IL-15 or IL-18 [6]. The importance of co-stimulation for IL-12 functionality is strong evidence for synergy between these cytokines. IL-12 is not normally detectable in healthy serum due to tight homeostatic regulation, which is beneficial given its highly pro-inflammatory nature. Furthermore, over-exposure to IL-12 can induce IL-12 hyporesponsiveness and downregulation of IFN-γ production by NK cells [13]. Therefore, use

of high concentrations of IL-12 *in vitro* may over-ride normal NK cell regulatory pathways, and the subsequent activation will not be representative of infection *in vivo*.

Similarly, the true synergies between combinations of cytokines will be obscured at high concentrations. For example, 10ng/ml of IL-12 [7] has been shown to drive IL-18Rα surface expression. This now needs to demonstrated with lower concentrations of IL-12 to provide stronger evidence for a role of IL-12-driven IL-18Rα upregulation in physiological IL-12/ IL-18 synergy. Likewise, 100ng/ml of IL-15 can also upregulate IL-18Rα [6], while 100IU/ml of IL-12 or IL-2 [4] can drive expression of the inducible IL-12R component (IL-12Rβ2), but these concentrations are unlikely ever achieved *in vivo*.

While titrating and combining IL-12, IL-15 and IL-18 was of interest to understand the basic kinetics of NK cell cytokine-driven activation very early in infection, I was more specifically concerned with how these innate cytokines interact with the adaptive arm of the immune response, as would occur during a recall response to a vaccine antigen. We therefore also investigated low concentrations of IL-12, IL-15 and IL-18 with increasing concentrations of IL-2 (as would be produced by CD4+ memory T cells) or CD16 crosslinking (as would occur in the context of IgG immune complexes).

The putative synergies with the antibody-dependent cellular cytotoxicity (ADCC) pathway, as modelled by CD16 crosslinking, were of particular interest since our data indicate that HCMV+ individuals have impaired CD107a upregulation in response to vaccine antigens as compared to HCMV- individuals (Chapter 4, [2]). I have previously considered the degranulation response, as indicated by CD107a upregulation, to be almost exclusively driven by IgG-CD16 crosslinking. It was therefore surprising that HCMV+ donors had reduced CD107a responses, given that they responded equally well to CD16 crosslinking with immobilised anti-CD16 (Chapter 4, [2]). This suggested that accessory cell cytokine signalling was synergising with the

IgG-CD16 pathway to drive degranulation. Additionally, while the IL-2 blocking data suggested degranulation was not dependent on IL-2, it remained unclear to what extent this pathway of NK cell activation was entirely T cell independent.

5.2 Methods

Apart from the CD4+ T cell depletions, the majority of the CD16 crosslinking assays and the H3N2 stimulations, which I performed alone, the work described in this chapter was jointly carried out with Asia-Sophia Wolf.

5.2.1 Study subjects

Volunteers were recruited from among staff and students at the London School of Hygiene and Tropical Medicine. All subjects gave written consent under a protocol for recruitment of blood donors approved by the LSHTM Ethics Committee (reference # 5520, Appendix III) to provide ≤ 50ml venous blood.

5.2.2 CD4+ T cell depletion

For assays that investigated the capacity of NK cells to respond to vaccine antigens in the absence of CD4+ T cells, peripheral blood mononuclear cells (PBMCs) were depleted of CD4+ cells prior to cell culture using CD4+ MACS microbeads (Miltenyi Biotec) and LS MACS Separation Columns (Miltenyi Biotec) according to the manufacturer's instructions. Briefly, thawed PBMC were incubated with CD4+ microbeads at 4°C for 15 minutes, then washed in MACS buffer (Table I, Chapter 2) before passing through an LS column in a QuadroMACS Separator magnet (Miltenyi Biotec), twice. Columns were rinsed with MACS buffer and the flow through was centrifuged to retrieve the CD4-depleted PBMC. CD4+ T cell depletion was confirmed by comparing CD3/CD4 profiles of undepleted and depleted PBMC from each donor. The average depletion efficiency was >99%, as illustrated in Figure 37.

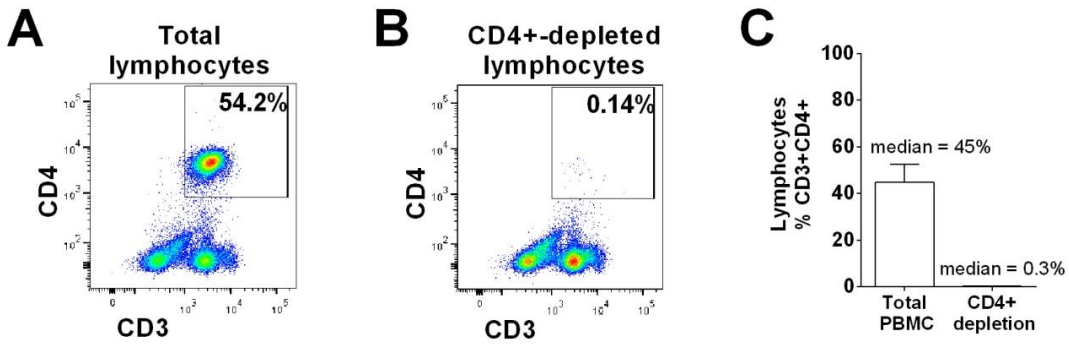

Figure 37. Confirmation of CD4+ T cell depletion with MACS microbeads. PBMC were depleted of CD4+ cells using anti-CD4 microbeads (Miltenyi) as per manufacturer's instructions. PBMC were stained for CD3 and CD4 expression to confirm removal of CD4+ T cells from the total lymphocyte population. Representative flow cytometry plots show CD3+CD4+ cells within the total lymphocyte population before **(A)** and after **(B)** depletion. This was consistent between all donors **(C)**. Bars represent medians and lines denote interquartile ranges. *n* = 4, data are from a single experiment.

5.2.3 PBMC preparation and culture

PBMC were isolated, cryopreserved, and thawed as described in Chapter 2.

PBMC (2 x 10^5/well) were cultured for 6 or 18 hours at 37°C at in 96-well U-bottom plates (Nunc) in complete medium with or without varying concentrations and combinations of recombinant human (rh) IL-2, rhIL-12, rhIL-15, rhIFN-α, rhIL-21 (all from PeproTech), rhIL-18 (R&D Biosystems) or inactivated influenza virus H3N2 (1µg/ml, NIBSC). GolgiStop (containing Monensin, 1/1500 concentration, BD Biosciences) and GolgiPlug (containing brefeldin A, 1/1000 final concentration, BD Biosciences) were added after 3 or 15 hours (in 6 or 18 hours cultures, respectively) in experiments where intracellular IFN-γ was a read-out. Similarly, anti-CD107a antibody (A488-conjugated, BD Biosciences) was included in the medium for the entirety of cell culture when CD107a upregulation was a read-out.

For activation via CD16 crosslinking, 96-well flat-bottom plates (Nunc) were coated with anti-human CD16 (BD Biosciences) or an isotype-matched control antibody (mIgG1κ, BD Biosciences) overnight at 4°C, as described in Chapter 3. Cells were harvested after 6 hours or 18 hours. GolgiStop, GolgiPlug and anti-CD107a were used as described above.

For experiments including CD4+ T cell-depleted PBMC, cells were cultured for 18 hours in complete medium supplemented with 10% FCS, rather than 10% AB plasma. GolgiStop, GolgiPlug and anti-CD107a were used as described above. Some conditions included stimulation with serum of known high-titre anti-pertussis toxin (PT) IgG titre (06/140, the WHO International Standard for Pertussis Antiserum [Human]; NIBSC). This serum, henceforth referred to as pertussis antiserum or anti-PT, was prepared from pooled re-calcified human serum and was reconstituted in sterile water in-house. To note, this is the same product that was used as the positive control for the anti-PT ELISAs described in Chapter 4.

5.2.4 Flow cytometry

PBMCs were stained in 96-well U-bottom plates as described in Chapter 2. The following monoclonal antibodies were used: anti-CD3-V500, anti-CD57-e450, anti-CD56-phycoerythrin(PE)-Cy7, anti-IFN-γ-allophycocyanin (APC), anti-CD107a-A488 (all BD Biosciences), anti-CD16-APC-H7, anti-CD16-APC, anti-CD25-PerCPCy5.5, anti-IL-18Rα-PE, anti-IL-18Rα-FITC, (all e-Biosciences). Anti-IL-12Rβ2 (R&D Systems) was conjugated to PerCP/Cy5.5 in-house (EasyLink PerCP/Cy5.5® Abcam).

5.2.5 Statistical analyses

Unless otherwise states, figures represent data from multiple experiments; sample sizes and number of experiments represented in each figure are described in legends. Flow cytometry and statistical analyses were performed as described in Chapter 2 and also as detailed in figure legends. Individual gated cell populations were excluded from analyses if they contained fewer than 100 cells. Paired Wilcoxon signed rank tests were used to compare responses between stimulation conditions and ANOVA tests for linear trend were used to analyse cytokine titrations. Formal tests for synergy using regression analysis with an interaction term, and linear regression adjusting for confounding factors were performed in STATA. All statistical tests were two-sided. **** $p \leq 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

5.3 Results

The data and analyses presented here are adapted and extended from Nielsen *et al*, manuscript submitted (Synergy between common γ chain family cytokines and IL-18 potentiates innate and adaptive pathways of NK cell activation, manuscript accepted).

5.3.1 Common γ chain family cytokines synergise with IL-18 to drive CD25 expression on NK cells

PBMC were stimulated with increasing concentrations of IL-2, IL-12, IL-15, IL-18, or IL-21, and NK cell surface expression of CD25 was measured after 6 or 18 hours (Figure 38B,C). Upregulation of CD25 is of interest as a marker of NK cell activation and, more specifically, increased sensitivity to IL-2; indeed, NK cell production of IFN-γ in response to picomolar levels of IL-2 has been shown to be CD25-dependent [14]. The highest cytokine concentrations tested reflect those widely used as positive controls by ourselves [1,2,15] and others; cytokines were then titrated to concentrations at least five-fold lower than the lowest previously described effective concentration.

IL-15 and IL-18 each, independently, drive CD25 expression in a dose- and time-dependent manner. Significant CD25 expression could be detected within 6 hours among cells cultured with cytokine concentrations as low as 0.75ng/ml IL-15 and 10ng/ml IL-18 (Figure 38B) but CD25 expression was markedly higher after 18 hours (consistent with time series data shown in Chapter 3) for both cytokines and evident at the lowest cytokine concentrations tested (0.75ng/ml IL-15 and 5ng/ml IL-18; Figure 38C). For IL-15, this is six-fold lower than the previously described minimal concentration [16,17] for upregulation of CD25, and ten- to 1000-fold lower than previously used concentrations of IL-18 [14,18]. Incubation of PBMC with IL-2, IL-12, and IL-21 induced minimal, albeit statistically significant, expression of CD25 on NK cells at 18 hours, but not at 6 hours.

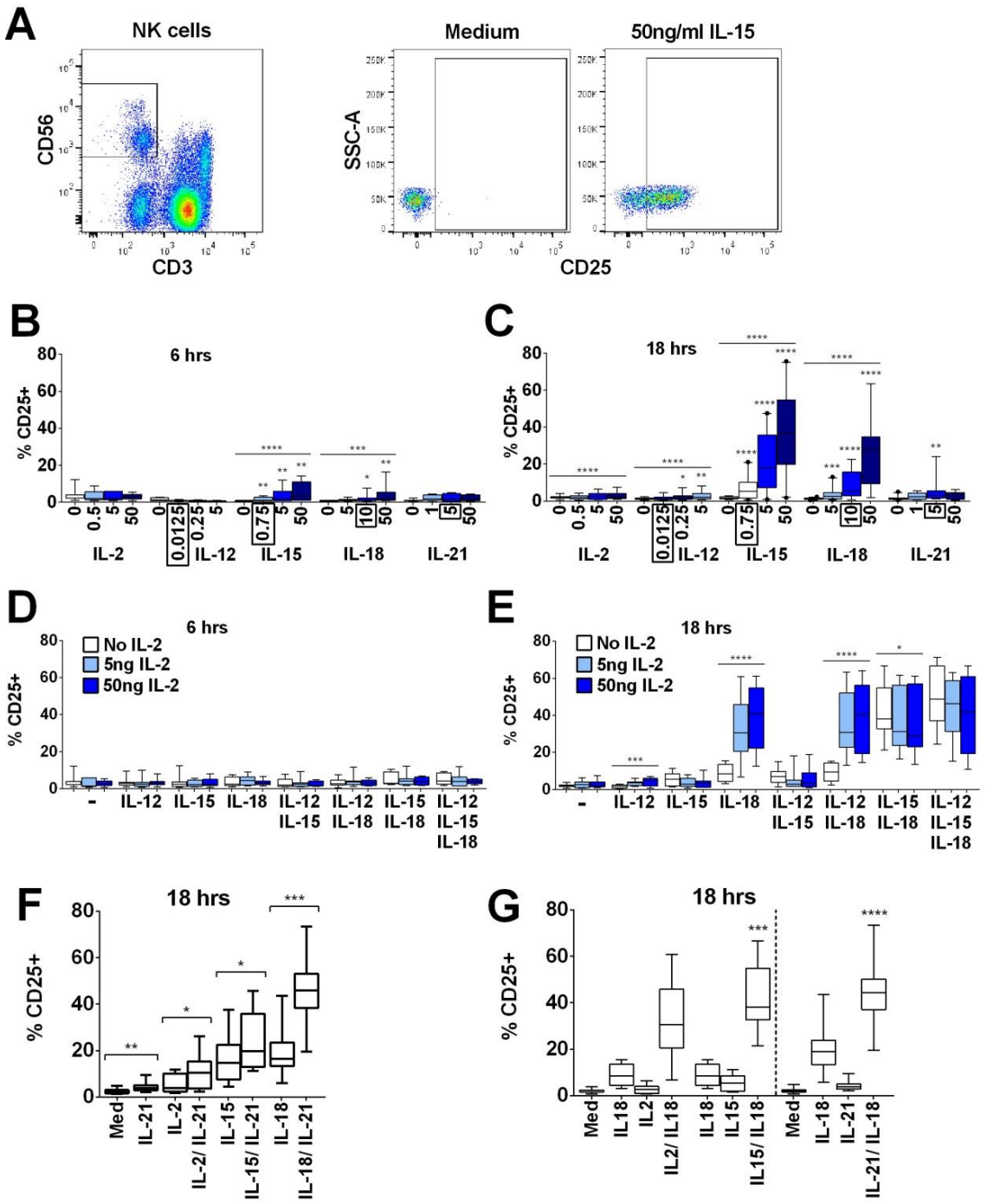

Figure 38. IL-18 and IL-15 both independently drive CD25 but interact differently with IL-2. PBMC were stimulated for 6 or 18 hours *in vitro* and upregulation of NK cell surface expression of CD25 was measured in response to medium alone (Med), IL-2, IL-12, IL-15, IL-18, or IL-21. Representative flow cytometry plots show gating of CD3-CD56+ NK cells and surface expression of CD25 on unstimulated and IL-15-stimulated NK cells (50ng/ml) **(A)**. CD25 expression on NK cells was measured after stimulation with Med, IL-2, IL-12, IL-15, IL-18, or IL-21 (concentrations ng/ml as labelled) for 6 hours **(B)** or 18 hours **(C)** *n* = 6-22, data from 2-6 experiments. Concentrations in boxes indicate those used in following graphs. CD25 expression on NK cells was also measured after stimulation with a titration of IL-2 (0, 5, 50ng/ml) in combination with IL-12 (12.5pg/ml), IL-15 (0.75ng/ml), and/or IL-18 (10ng/ml) for 6 hours **(D)** or 18 hours **(E)** *n* = 7-8, data from two experiments. CD25 expression on NK cells was also measured following stimulation with 5ng/ml IL-21 in combination with 5ng/ml IL-2, 0.75ng/ml IL-15, or 10ng/ml IL-18 after 18 hours **(F)** *n* = 8, data from a single experiment. CD25 expression after stimulation for 18 hours **… (continued on page 164)**

(continued from page 163)
… with a combination of IL-18 (10ng/ml) and common γ chain cytokines was summarised to facilitate comparison between IL-2 (5ng/ml), IL-15 (0.75ng/ml; both from D) and IL-21 (5ng/ml; from F) **(G)** *n* = 7-8, data from 1-2 experiments. Box plot whiskers show the 5-95th percentile range. Data were analysed using paired Wilcoxon signed-rank tests (B-C: asterisks without lines; lowest concentration compared to Med; F: capped lines) or ANOVA tests for linear trend for trend analysis across increasing cytokine concentrations including Med (B-E, uncapped lines). ****$p \leq 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

To investigate potential synergies between cytokines in driving CD25 expression on NK cells, PBMC were stimulated with combinations of IL-12, IL-15 and IL-18, with or without varying concentrations of IL-2, to model early NK cell activation in response to primary pathogen infection (innate cytokines only, no IL-2) and secondary infection (innate cytokines plus IL-2 from memory CD4+ T cells). We selected the lowest concentrations of IL-12 and IL-15 that had been tested singly (12.5pg/ml and 0.75ng/ml, respectively) and, for consistency with our own previously published work [1,2,15], we used the middle concentration of IL-18 (10ng/ml). The middle concentration of IL-21 (5ng/ml), an adaptive common γ chain (γ_c) cytokine, was selected to permit later comparisons with IL-2.

Consistent with the data presented in Figure 38B, CD25 expression was very low after 6 hours and there was no significant evidence of synergism between cytokines (35D). However, after 18 hours, the data clearly showed synergy between IL-18 and IL-2 in driving NK cell CD25 expression (trend analysis $p < 0.0001$ for IL-18 in combination with increasing concentrations of IL-2) with 5ng/ml IL-2 in combination with 10ng/ml IL-18 giving CD25 expression levels equivalent to those seen with 50ng/ml IL-18 alone (Figure 38C,E). Although adding IL-12 to a cocktail of IL-2 plus IL-18 did not further enhance CD25 expression, including a low concentration of IL-12 (0.0125ng/ml) in the cultures did permit detection of a modest IL-2 dose-response (Figure 38E) and much higher, though less physiological, concentrations of IL-12 (1-10ng/ml) do synergise with IL-18 to drive CD25 expression (Figure 39) [14,19]. There was

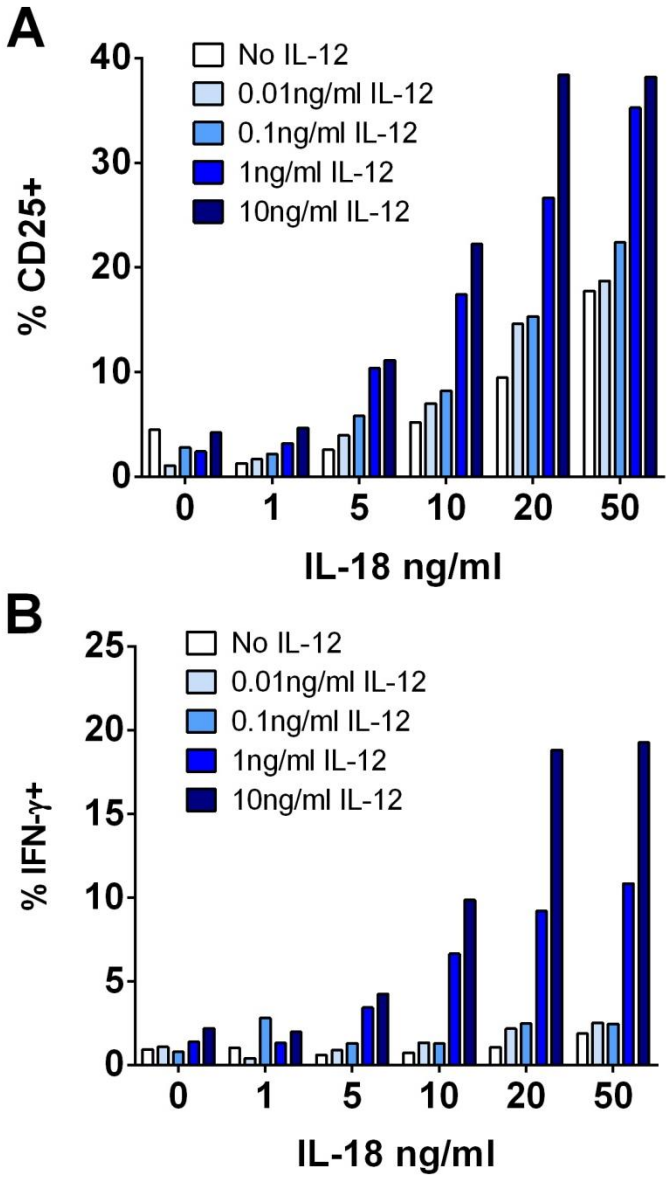

Figure 39. IL-12 and IL-18 synergised to drive enhanced NK cell CD25 and IFN-γ responses in dose-dependent manner. PBMC were stimulated for 18 hours with medium alone, or increasing concentrations of IL-12 and/or IL-18 (as labelled on graphs). NK cell surface expression of CD25 (**A**) and IFN-γ (**B**) was measured after 18 hours. *n* = 2, data from a single experiment.

also strong evidence that IL-15 synergises with IL-18 to enhance NK cell CD25 expression (Figure 38G, test for interaction IL-15 and IL-18, $p = 0.009$).

By contrast, IL-15-driven CD25 upregulation was partially inhibited by IL-2: there was a trend for the proportion of NK cells expressing CD25 to decrease with increasing concentrations of IL-2 in all cytokine combinations that included IL-15, with a statistically significant impact observed in NK cells stimulated with IL-15 plus IL-18 (median without IL-2 = 38.1%, vs. median with high concentration IL-2 = 29.0%; linear test for trend, $p = 0.040$; Figure 38E).

In a separate set of experiments, we also tested IL-21 and IFN-α for their ability to synergise with IL-2, IL-15 and IL-18 to drive CD25 expression on NK cells. There was no evidence that IFN-α alone induced CD25 expression, nor did it enhance CD25 expression in combination with other cytokines (Figure 40A-C). However, IL-21 in combination with IL-2, IL-15 or, in particular, IL-18 significantly enhanced CD25 expression compared to these cytokines alone (Figure 38F). Indeed, there was clear evidence of synergy between IL-21 and IL-18 driving CD25 expression (Figure 38G, test for interaction IL-21 and IL-18, $p < 0.0001$).

In summary, these data indicate that at least three different cytokines (IL-15, IL-2, IL-21) that signal via the common gamma chain (γ_c; CD132) can individually synergise with the IL-18 pathway leading to rapid upregulation of CD25 expression on NK cells, and at much lower cytokine concentrations than previously appreciated (Figure 38G). As IL-15 and IL-18 are produced primarily by dendritic cells, monocytes and macrophages, and as IL-2 and IL-21 are primarily T cell-derived, these combinations of cytokines allow for very early NK cell activation – when cytokine concentrations are still extremely low – via both innate and adaptive immune pathways. Moreover, there is evidence of homeostatic regulation of NK cell activation via γ_c cytokines, as illustrated by inhibition of IL-15-driven CD25 upregulation by IL-2.

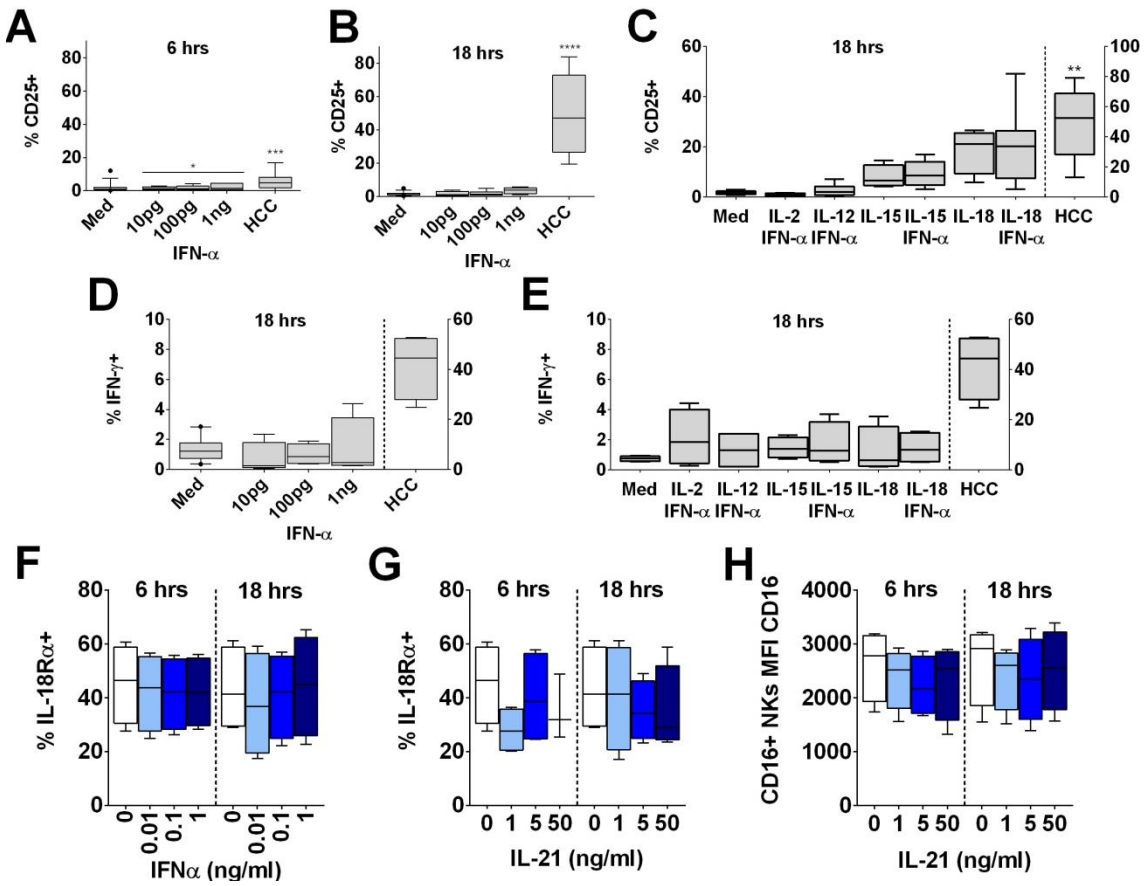

Figure 40. IFN-α or IL-21 alone have minimal effects on NK cell responses. PBMC were stimulated with increasing concentrations of IFN-α or IL-21. NK cell surface expression of CD25 was measured in response to medium alone (Med), 10pg, 100pg or 1000pg/ml IFN-α alone, or high concentration of cytokines (HCC: 5ng/ml IL-12, 50ng/ml IL-18), at 6 hours **(A)** and 18 hours **(B)**, and to combinations of IFN-α (10pg/ml) and IL-2 (5ng/ml), IL-12 (12.5pg/ml), IL-15 (0.75ng/ml) or IL-18 (10ng/ml) after 18 hours **(C)** *n* = 8, data from two experiments. Likewise, intracellular production of IFN-γ was measured after 18 hours in response to medium alone, IFN-α alone, or HCC **(D)** or in combination with IL-2, IL-12, IL-15 or IL-18 as previously mentioned **(E)** *n* = 4, data from a single experiment. Expression of IL-18Rα was also measured at 6 hours and 18 hours in response to IFN-α titration **(F)** and IL-21 titration **(G)** at the cytokine concentrations indicated on the graphs *n* = 4, data from a single experiment. Finally, CD16 MFI of CD56dimCD16+ NK cells was measured after 6 or 18 hours **(H)** *n* = 4, data from a single experiment.

5.3.2 Common γ chain cytokines synergise with IL-18 to drive rapid and extensive IFN-γ production by NK cells

Upregulation of CD25 primes NK cells for enhanced subsequent responses to IL-2 but is not, in itself, a read-out of NK cell effector function. We have therefore characterised the effect of combining low concentrations of different cytokines on IFN-γ production, assessed by intracellular staining after incubation of PBMC with increasing concentrations of individual cytokines or cytokine combinations (Figure 41).

Increasing concentrations of IL-2, IL-12, IL-15 or IL-18 (but not IL-21) each, individually, induced significant IFN-γ production by NK cells at 18 hours, although the proportions of IFN-γ+ cells rarely exceeded 10% even at the highest cytokine concentrations (Figure 41B). However, combining IL-18 (at a concentration of 10ng/ml), with as little as 5ng/ml IL-2 (which alone did not drive IFN-γ) not only induced IFN-γ in much higher proportions of NK cells (>20%) but did so within 6 hours of incubation (Figure 41C). Whilst this interaction appears additive at 6 hours (test for interaction, p = 0.220) by 18 hours the interaction is highly synergistic (test for interaction, p = 0.006), possibly as a result of IL-18 induced upregulation of the high affinity IL-2R (CD25, as shown in Figure 38).

In contrast to what we observed for CD25 expression, there was no evidence of antagonism or competition between γ_c cytokines in their induction of NK cell IFN-γ. On the contrary, there was evidence of additive or synergistic interactions between γ_c cytokines with increasing concentrations of IL-2 modestly but significantly enhancing NK cell IFN-γ responses to IL-15 with IL-18 (Figure 41C-D). Low concentrations of IL-15 (Figure 41C-D) and IL-21 (Figure 41E) also enhanced IL-18-induced NK cell IFN-γ production, but to a lesser extent than IL-2 (Figure 41C,D,F). Although IL-15 plus IL-18 has previously been shown to enhance NK cell IFN-γ, as measured by ELISA, the effects described here were apparent at an IL-15 concentration (0.75ng/ml) markedly lower than previously described (5ng/ml) [16].

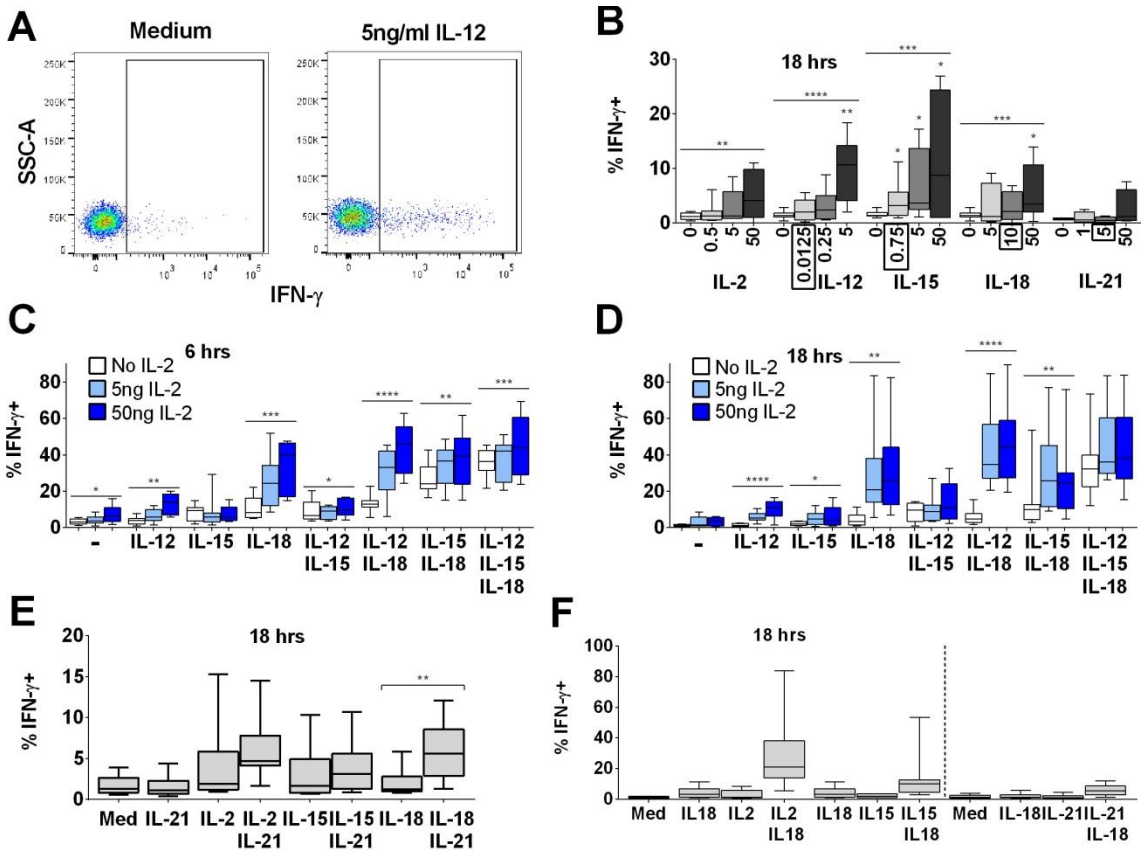

Figure 41. IL-15 and IL-18 can synergise to drive IFN-γ in absence of IL-12 or IL-2. PBMC were stimulated for 6 or 18 hours *in vitro* and production of intracellular IFN-γ by NK cells was measured in response to Med (medium alone), IL-2, IL-12, IL-15, IL-18, or IL-21. Representative flow cytometry plots show gating of CD3-CD56+ NK cells and percentage positive for intracellular IFN-γ on unstimulated and IL-12-stimulated NK cells (5ng/ml) **(A)**. IFN-γ production by NK cells was measured after stimulation with Med, IL-2, IL-12, IL-15, IL-18, or IL-21 (concentrations ng/ml as labelled) for 18 hours **(B)** *n* = 4-9, data from 1-3 experiments. Concentrations in boxes indicate those used in following graphs. IFN-γ production by NK cells was also measured after stimulation with a titration of IL-2 (0, 5, 50ng/ml) in combination with IL-12 (12.5pg/ml), IL-15 (0.75ng/ml), and/or IL-18 (10ng/ml) for 6 hours **(C)** or 18 hours **(D)** *n* = 7-8, data from two experiments. IFN-γ production by NK cells was also measured following stimulation with 5ng/ml IL-21 in combination with 5ng/ml IL-2, 0.75ng/ml IL-15, or 10ng/ml IL-18 after 18 hours **(E)** *n* = 8, data from a single experiment. IFN-γ expression after stimulation for 18 hours with a combination of IL-18 (10ng/ml) and common γ chain cytokines was re-plotted to facilitate comparison between IL-2 (5ng/ml), IL-15 (0.75ng/ml; both from D), and IL-21 (5ng/ml; from E) for 18 hours **(F)** *n* = 7-8, data from 1-2 experiments. Box plot whiskers show the 5-95th percentile range. Data were analysed using paired Wilcoxon signed-rank tests (B: asterisks without lines; lowest concentration compared to Med; E: capped lines) or ANOVA tests for linear trend for trend analysis across increasing cytokine concentrations including Med (B-D, uncapped lines). ****$p \leq 0.0001$, *** $p < 0.001$, **$p < 0.01$, *$p < 0.05$. $n \geq 8$, other than for IL-21 titration (B) where $n = 4$.

Again, as for CD25 expression, we found no evidence of a role for IFN-α in NK cell IFN-γ production (Figure 40D-E); this is in contrast to published data [5]. Low concentrations of IL-12 (0.0125ng/ml) — alone or in combination with IL-2 or IL-21 — had minimal effects on IFN-γ production (Figure 41C-E), but did enhance IFN-γ production in combination with IL-15 and IL-18 at later time points. High concentrations of IL-12 (≥ 1ng/ml) synergised strongly with IL-18 to drive both IFN-γ and CD25, although we suggest that these do not reflect physiological conditions (Figure 39) [6-8,14,18]. Overall, however, as little as 5ng/ml IL-2 in combination with low concentrations of IL-18 (10ng/ml) and IL-12 (12.5pg/ml) was the optimal combination for NK cell IFN-γ induction at 18 hours.

In summary therefore, γ_c cytokines (IL-2, IL-15 and IL-21) in combination with IL-18 induce very rapid and extensive IFN-γ production by NK cells (Figure 41F). Although IL-2 seems to be the most potent of these, at least at the cytokine concentrations tested, the ability of IL-15 to augment IFN-γ production offers a route for rapid, innate activation of NK cells prior to the differentiation of IL-2 secreting T cells. Of interest, given the very large body of work describing IFN-γ induction by combinations of IL-12 and IL-18, γ_c cytokines synergise with IL-18 at extremely low concentrations. It is possible therefore that, *in vivo*, IL-12 may contribute to NK cell IFN-γ production when γ_c cytokines are lacking, such as during primary exposure (when IL-2 from antigen-specific T cells may be limiting) or later in infection when IL-15 signalling is reduced by changes in receptor expression [20,21].

5.3.3 IL-18 signalling sustains IL-18Rα expression on NK cells

As IL-18 alone is able to induce both CD25 and IFN-γ expression within 6 hours (Figures 38B, 41C), we hypothesised that maintaining the capacity for IL-18 signalling might be required for optimal NK cell activation. Thus the sustained or enhanced expression of the IL-18R may contribute to the synergy between IL-18 and γ_c cytokines. To determine whether, and if so which, cytokines regulate IL-18R expression, NK cell surface expression of IL-18Rα (CD218a, the

receptor component required for signalling [22]) was measured after 6 hours or 18 hours of PBMC culture with IL-2, IL-12, IL-15, IL-18, IL-21 or IFN-α, alone and in combination (Figures 40 and 42). It was immediately obvious that resting levels of IL-18Rα expression are extremely variable between donors with the proportion of resting NK cells expressing the receptor varying from approximately 20% to greater than 80% (Figure 42B-C).

Polymorphisms affecting DNA methylation within the promoter region of *IL18R1*, the gene encoding IL-18Rα, and subsequent transcription of the gene have been reported and may in part explain this variation [23,24] and we have previously observed lower levels of IL-18Rα expression in human cytomegalovirus seropositive (HCMV+) than in HCMV seronegative (HCMV-) individuals (Chapter 4, [2]). Despite this inter-individual variation, resting levels of IL-18Rα expression are very high in comparison to resting levels of the high affinity IL-2R (as defined by expression of the IL-2Rα chain, CD25; see Figure 38B-C) and fully functional IL-12R (as defined by expression of IL-12R-β2; Figure 43) and may explain the very rapid (within 6 hours) NK cell response to exogenous IL-18 (Figures 38B, 41C). Indeed, we observed a weak but statistically significant correlation between resting levels of NK cell IL-18Rα expression and upregulation of CD25 following IL-18 stimulation (n = 18, 50ng/ml IL-18, linear regression in STATA [adjusting for use of PE- and FITC-conjugated anti-IL-18Rα)] $R^2 = 0.241$, $p = 0.046$).

Contrary to previously published data indicating that IFN-α [25] and IL-12 [6-8,25] can individually induce IL-18R mRNA [6,25], IL-18Rα protein expression [7], or IL-18R expression [8] in human NK cells, and that IL-12/STAT4 signalling induces IL-18Rα expression in mice [26,27], we found no increase in surface expression of IL-18Rα in response to increasing concentrations of either IFN-α (Figure 40F) or IL-12 (Figure 42B) with or without IL-2 (Figure 42C). There are several likely explanations for this discrepancy. For example, in previous studies exogenous IL-2 was routinely added to NK cell cultures and IFN-α was used at much higher concentrations [25]; NK cells were stimulated with very high concentrations of IL-12 [6,7]; IL-18R mRNA was

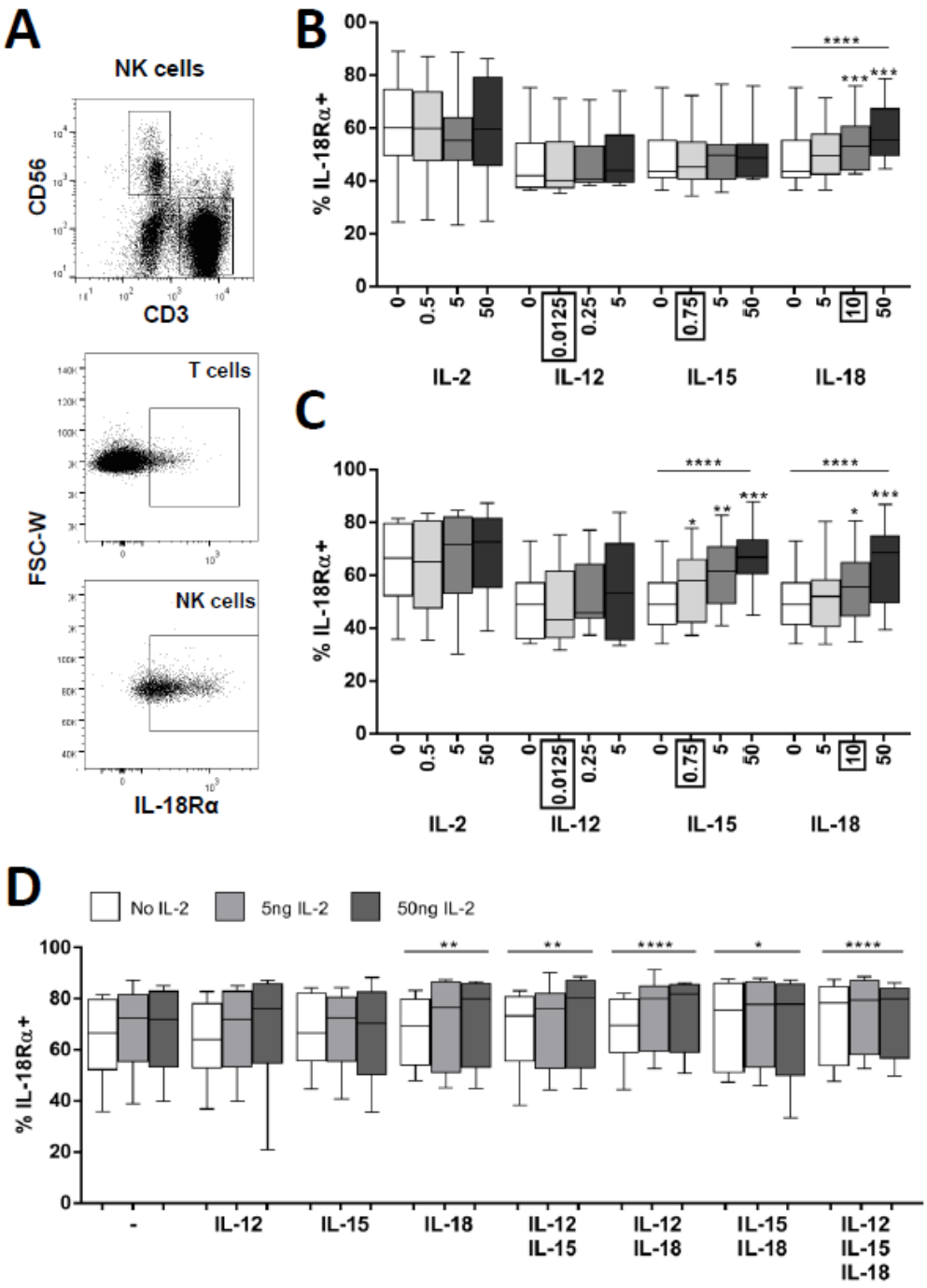

Figure 42. Positive feedback from IL-18 induces IL-18Rα. PBMC were stimulated for 6 or 18 hours *in vitro* and changes in NK cell surface expression of IL-18Rα was measured in response to medium alone (Med), IL-2, IL-12, IL-15, IL-18, or IL-21. Representative flow cytometry plots show gating of CD3+ T cells, CD3-CD56+ NK cells, and surface expression of IL-18Rα on unstimulated T cells and NK cells for IL-18Rα-FITC (N.B. as used in D; IL-18Rα-PE used in B-C) **(A)**. IL-18Rα expression on NK cells was measured after stimulation with Med, IL-2, IL-12, IL-15, IL-18, or IL-21 (concentrations ng/ml as labelled) for 6 **(B)** or 18 hours **(C)**, *n* = 7-11 data from 1-2 experiments. Concentrations in boxes indicate those used in following graphs. IL-18Rα expression on NK cells was also measured after stimulation with a titration of IL-2 (0, 5, 50ng/ml) in combination with IL-12 (12.5pg/ml), IL-15 (0.75ng/ml), and/or IL-18 (10ng/ml) after 18 hours **(D)**, *n* = 8, data from two experiments. Box plot whiskers show the 5-95th percentile range. Data were analysed using paired Wilcoxon signed-rank tests (B-D: asterisks without lines; compared to Med) or ANOVA tests for linear trend for trend analysis across increasing cytokine concentrations including Med (B-D, uncapped lines).
****$p \leq 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

assessed rather than IL-18R protein [6,25]; NK cells were purified by positive selection which may, in itself, contribute to subsequent activation [6,7]; or components of the IL-18R other than IL-18Rα were measured, such as AcPL [6,8,25]. Nevertheless, our data suggest that at physiological concentrations, IL-2, IL-12 and IFN-α have little, if any, effect on IL-18Rα expression. We also observed no effect of IL-21 on IL-18Rα expression (Figure 40G).

By contrast, we found clear evidence of concentration-dependent upregulation of NK cell IL-18Rα expression in response to IL-15 alone and IL-18 alone (Figure 42B,C). We observed 10 ng/ml of IL-18 was sufficient to upregulate IL-18Rα within 6 hours (Figure 42B, p=0.001) and this effect was sustained at 18 hours (Figure 42C, p = 0.0003). IL-15 had no effect on IL-18Rα expression at 6 hours, but as little as 0.75ng/ml IL-15 was sufficient to upregulate IL-18Rα expression by 18 hours (Figure 42B-C, p = 0.019). Overall, IL-15 and IL-18 each increased the proportion of NK cells expressing IL-18Rα by approximately 15% (median percentage of IL-18Rα+ NK cells: 50.1% medium only; 64.6% with 50ng IL-18; 66.8% with 50ng IL-15). The ability of IL-18 to rapidly augment expression of its own receptor is suggestive of a positive feedback loop, allowing for enhanced IL-18 signalling, continued synergism with other signalling pathways, and efficient induction of NK cell effector functions in the first few hours of infection.

We next considered whether other cytokines might synergise with IL-18 to further enhance IL-18Rα expression. Increasing concentrations of IL-2, either alone or in combination with IL-12 or IL-15 had no significant effect on IL-18Rα expression at either 6 hours (not shown) or 18 hours (Figure 42D). However, IL-2 modestly but significantly enhanced the effects of IL-18 in a dose dependent manner and there was an additive effect of combining IL-15 and IL-18 in the absence of IL-2 (Figure 42D). Addition of other cytokines to the IL-18/ IL-2 combination did not further enhance IL-18Rα expression. Although, at the low cytokine concentrations used in these experiments (0.0125ng/ml IL-12, 10ng/ml IL-18), we saw no additive or synergistic effect

of adding IL-12 to IL-18, at much higher concentrations (5ng/ml IL-12 and 50ng/ml IL-18) we did observe a significant ($p < 0.0001$) additive effect of these two cytokines increasing IL-18Rα expression (data not shown).

These data support my assertion that IL-18 is a key cytokine in initiating and sustaining NK cell responses under physiologically relevant conditions such as very early infection and that NK cell responses that can be induced with very high (non-physiological) cytokine concentrations in vitro may not be relevant *in vivo*.

In summary therefore, low concentrations of IL-18 rapidly and significantly upregulate the IL-18Rα subunit and this effect is augmented by low concentrations of IL-15 and (more substantially) by IL-2. Given that IL-18 alone is sufficient to induce expression of the high affinity IL-2R (Figure 38B-C), it seems that IL-18 and IL-2 synergistically and reciprocally upregulate their own and each other's receptors in a potent positive feedback loop. The minimal role of low concentrations of IL-12 in this process may explain the limited synergies of exogenous IL-12 in the early NK cell IFN-γ response (Figure 41).

For completeness, we also examined expression of IL-12Rβ2 in response to single cytokines or cytokine combinations (Figure 43). The proportion of NK cells expressing IL-12Rβ2 was transiently (seen at 6 hours but not at 18 hours) and very modestly enhanced by 10-50ng/ml IL-18 and, in a slightly more sustained fashion, by 50ng/ml IL-15, but the biological relevance of such small effects is unclear. There was no effect of exogenous cytokines on IL-12Rβ2 expression at the level of individual cells (as measured by MFI).

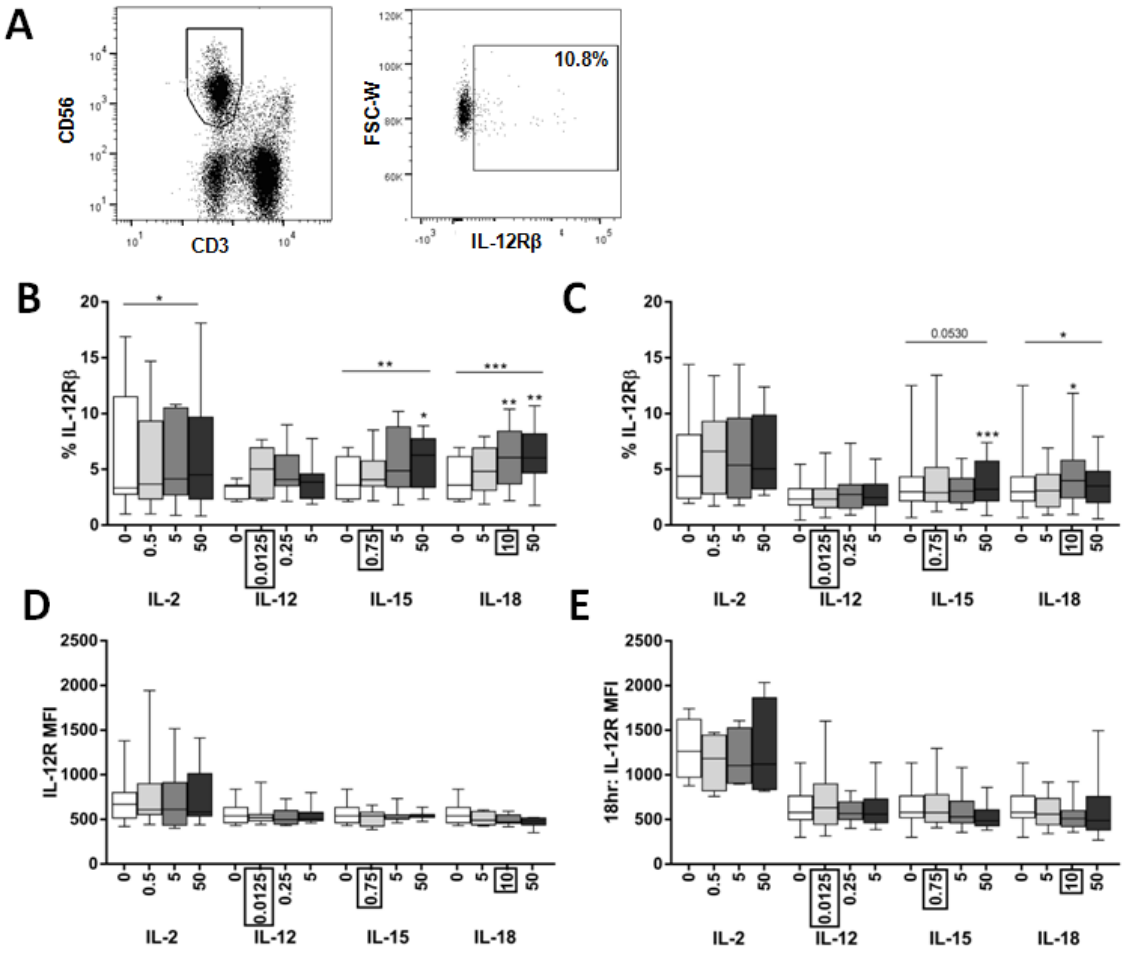

Figure 43. Low level IL-12Rβ2 upregulation following IL-15 or IL-18 stimulation. PBMC were stimulated for 6 or 18 hours *in vitro* and changes in NK cell surface expression of IL-12Rβ2 was measured in response to medium alone (Med), IL-2, IL-12, IL-15, or IL-18. Representative flow cytometry plots show gating of CD3-CD56+ NK cells and surface expression of IL-12Rβ2 on unstimulated cells **(A)**. IL-12Rβ2 expression on NK cells was measured after stimulation with Med, IL-2, IL-12, IL-15, IL-18 (concentrations in ng/ml as labelled) after 6 hours **(B)** n = 7-16, data from 1-3 experiments or 18 hours **(C)** n = 8-18, data from 2-3 experiments, with mean fluorescence intensity (MFI) of IL-12Rβ2 expression on NK cells for the same experiments at 6 hours **(D)** and 18 hours **(E)**. NB: IL-2 titrations were performed using a different batch of anti-IL-12Rβ2-PerCP/Cy5.5 conjugated antibody. Data were analysed using paired Wilcoxon signed-rank tests (B-C: asterisks without lines; compared to Med) or ANOVA tests for linear trend for trend analysis across increasing cytokine concentrations including Med (B-C, uncapped lines). ***$p \leq 0.001$, **$p < 0.01$, *$p < 0.05$.

5.3.4 IL-18 synergises with FcγRIII (CD16) signalling to augment NK cell mediated antibody-dependent cellular cytotoxicity

After vaccination, or upon secondary infection, circulating antigen-antibody complexes binding to FcγRIII (CD16) on NK cells can mediate killing of infected cells via ADCC. As our data suggest that IL-18, in concert with γ_c cytokines, enhances adaptive as well as innate pathways of NK cell activation, we wanted to test whether ADCC could be augmented by very low levels of NK cell activating cytokines, as would be present at the site of infection (Figure 44).

We found that IL-18, but not IL-12, IL-15, or IL-21, induced rapid (within 6 hours) and sustained (persists at 18 hours), concentration-dependent downregulation of CD16 expression at the NK cell surface; cells substantially lost CD16 expression within 6 hours in the presence of 10ng/ml IL-18 (Figure 44A-B, Figure 40H). We have previously observed that crosslinking of CD16 with plate-bound anti-CD16 antibody leads to loss of CD16 from the NK cell surface (Goodier *et al*, unpublished data), and this is consistent with previous reports of CD16 downregulation following CD16 ligation [28]. Here we observe that the inherent capacity of IL-18 to reduce CD16 expression synergises with the effects of CD16 crosslinking such that after 6 and 18 hours, residual CD16 expression is lower when NK cells are cultured with 10ng/ml IL-18 plus plate bound anti-CD16 than when they are cultured with either anti-CD16 or IL-18 alone (Figure 44C). This is true whether cells are cultured in 10% pooled human AB plasma (as per my standard assay protocol) or 10% FCS (Figure 45).

Taken together with published data indicating that downregulation of CD16 on CD56dim NK cells in response to either CD16 crosslinking or to very high concentrations of IL-12 (10ng/ml) plus IL-18 (100ng/ml) can be blocked with a specific inhibitor specific of the metalloprotease (MMP) ADAM-17 [28], these data raise the interesting hypothesis that the IL-18 and the CD16 signalling pathways may converge to induce metalloprotease (MMP)-mediated cleavage of CD16 from

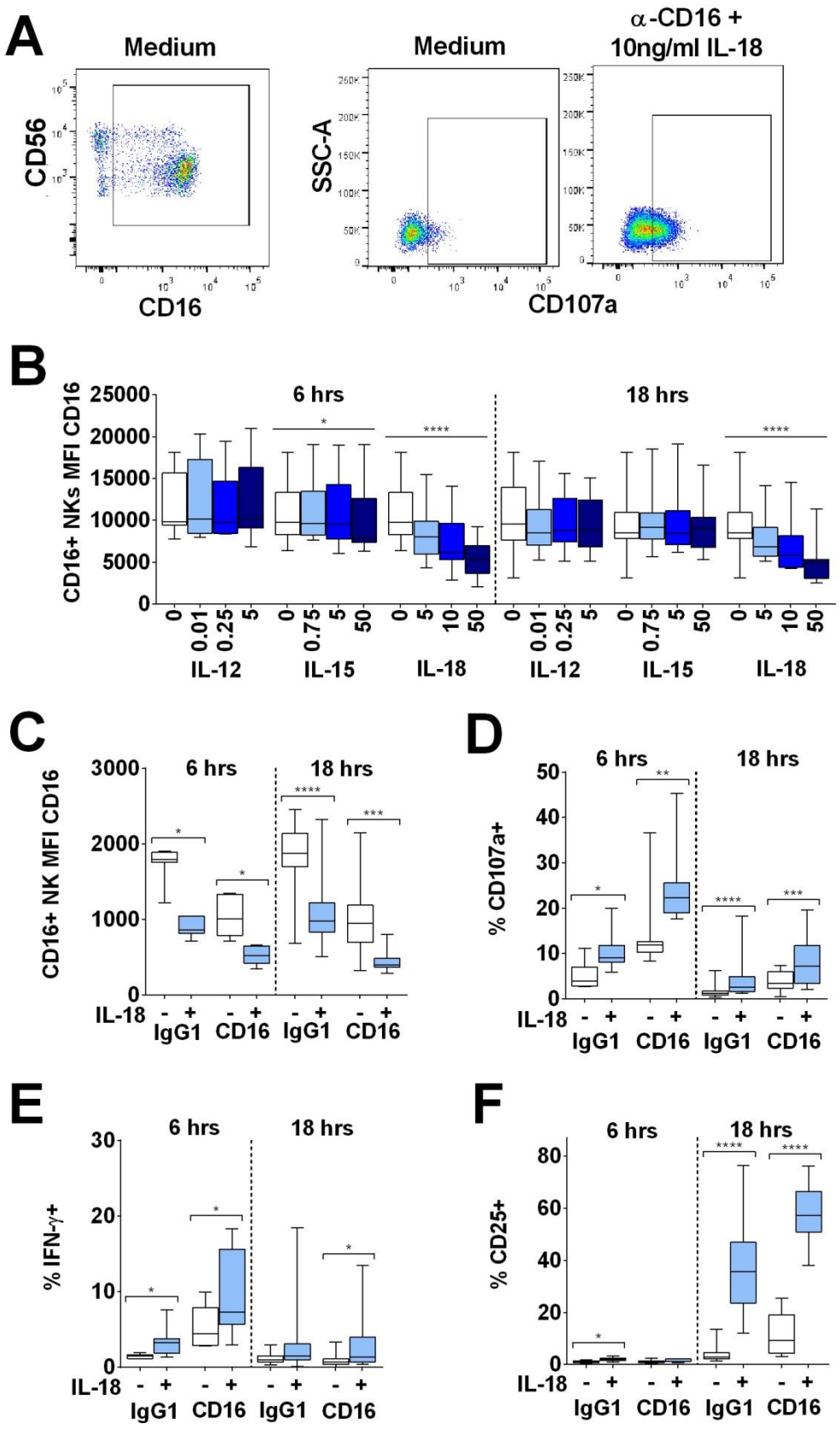

Figure 44. IL-18 enhances responses to CD16 crosslinking, while simultaneously driving CD16 downregulation. PBMC were stimulated for 6 or 18 hours *in vitro* and changes in CD16 MFI (mean fluorescence intensity) of CD16+ NK cells were measured in response to medium alone (Med), IL-12, IL-15, or IL-18. Representative flow cytometry plots show gating of CD3-CD56+ NK cells, and surface expression of CD107a or CD16 on unstimulated cells, or CD107a on NK cells activated with CD16 crosslinking and 10ng/ml IL-18 **(A)**. **… (continued on page 178)**

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… CD16 MFI on CD56dimCD16+ NK cells was measured after stimulation with Med, IL-12, IL-15, or IL-18 (concentrations ng/ml as labelled) for 6 or 18 hours **(B)** *n* = 9-13, data from 2-3 experiments. For the crosslinking assays, PBMC were stimulated for 6 or 18 hours *in vitro* with Med, α-CD16 or its IgG1 isotype control, with (+) or without (-) 10ng/ml IL-18. CD16 MFI of CD56dimCD16+ NK cells was measured after 6 or 18 hours **(C)**, *n* = 7-16, data from 1-2 experiments. Surface expression of CD107a **(D)**, intracellular IFN-γ **(E)**, and CD25 **(F)** was measured on NK cells after 6 or 18 hours, *n* = 7-16, data from 1-2 experiments. Box plots show the 5-95th percentile range. Data were analysed using paired Wilcoxon signed-rank tests (C-F: capped lines) or ANOVA tests for linear trend for trend analysis across increasing cytokine concentrations including Med (B, uncapped lines). ****$p \leq 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

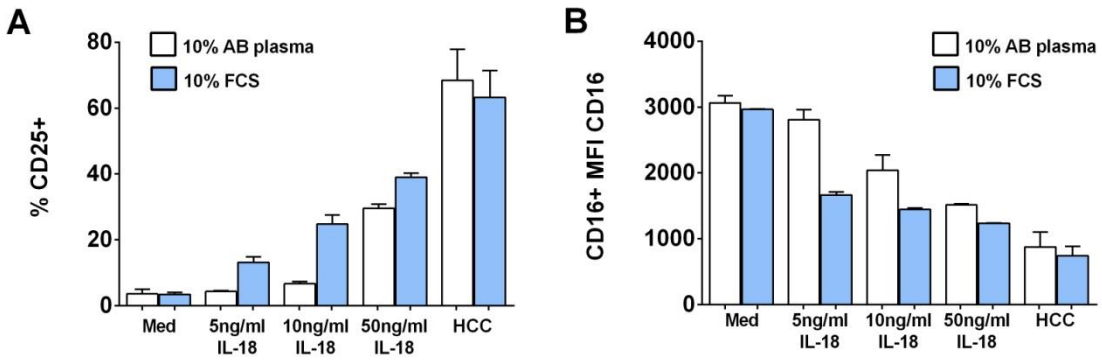

Figure 45. IL-18 drives downregulation of CD16 in the absence of IgG. PBMC were stimulated for 18 hours with medium alone, 5/10/50ng/ml IL-18 or a high high concentration of cytokines (HCC: 5ng/ml IL-12, 50ng/ml IL-18) in medium supplemented with 10% pooled human AB plasma or 10% FCS. Dose-dependent NK cell activation in AB and FCS was confirmed by upregulation of surface CD25 **(A)** alongside downregulation of CD16 on CD16+ NK cells, as measured by MFI **(B)**. Bars represent medians and lines denote interquartile ranges. *n* = 2, data from a single experiment.

the cell surface. The reciprocal impact of CD16-driven activation on surface IL-18Rα was much less clear, with only a slight trend towards negative feedback of CD16 crosslinking on IL-18Rα expression (Figure 46).

Using crosslinking of cell surface CD16 with plate-bound anti-CD16 antibody as a model of ADCC [28], we next determined the effects of low concentrations of IL-18 on NK cell cytotoxicity (assessed using the CD107a degranulation assay [29,30]) as well as on CD25 and IFN-γ responses. Despite the very rapid downregulation of CD16 by IL-18 and CD16 crosslinking (Figure 44B-C), we observed that as little as 10ng/ml IL-18 markedly and very rapidly (within 6 hours) augmented NK cell degranulation and IFN-γ production in the presence of anti-CD16 antibody (Figure 44D-E).

Furthermore, IL-18 synergised with anti-CD16 to enhance CD25 expression at 18 hours (Figure 44F; test for interaction, p = 0.005). These data demonstrate that IL-18 can substantially enhance NK cell ADCC responses and also support the idea that downregulation of CD16 expression is a consequence of activation of signalling pathways downstream of CD16.

To validate this apparent interaction between IL-18 and CD16, PBMC were incubated for 18 hours with or without whole, inactivated H3N2 influenza virus in the presence of plasma that had previously been shown to contain anti-H3N2 IgG. NK cell degranulation, CD25, and IFN-γ responses to H3N2 immune complexes were compared in the presence or absence of exogenous IL-18 (Figure 47). As reported above, IL-18 alone induced modest increases in CD107a, CD25 and IFN-γ expression.

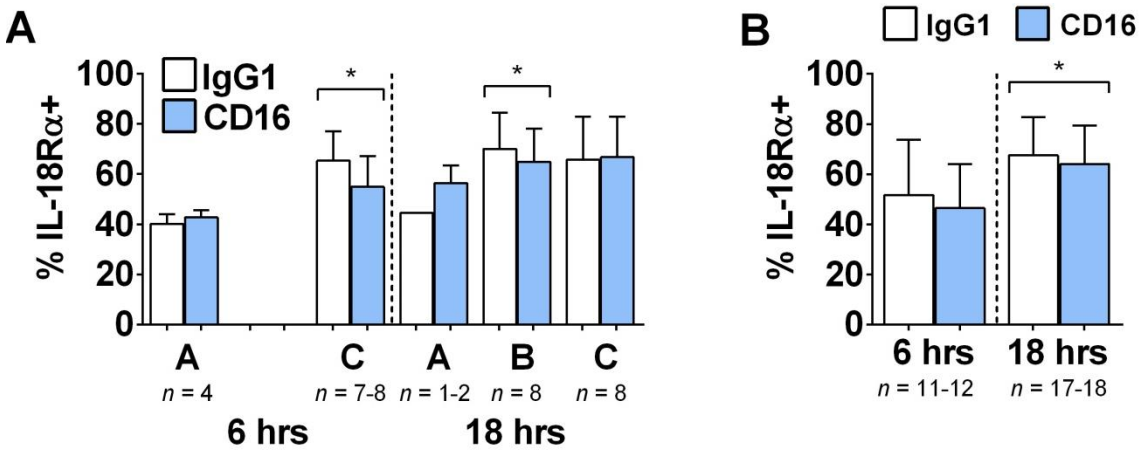

Figure 46. CD16 crosslinking may minimally downregulate IL-18Rα surface expression. PBMC were stimulated for 6 or 18 hours *in vitro* and changes to total NK cell IL-18Rα surface expression were measured in response to CD16 crosslinking, or IgG isotype control in three separate experiments: A, B (18 hours only), and C. Experiments were analysed separately **(A)** or together **(B)** using paired Wilcoxon signed-rank tests. Bars denote medians and lines indicate interquartile ranges. *$p < 0.05$. Sample sizes are annotated below axis.

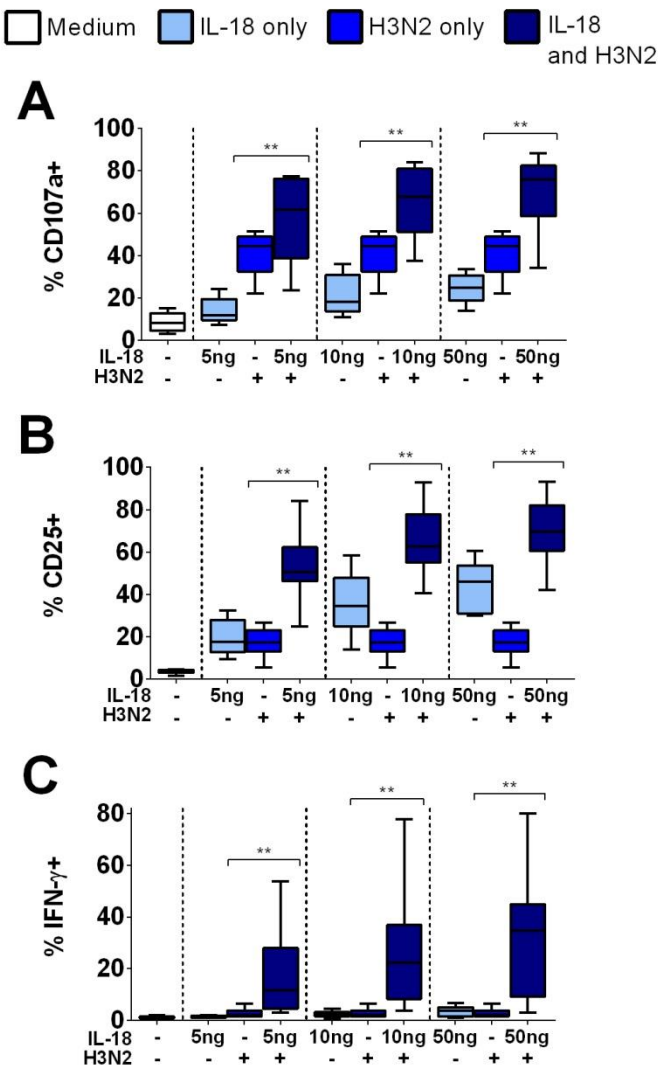

Figure 47. Antibody and IL-18 synergism in response to H3N2. PBMC were stimulated for 18 hours *in vitro* medium alone (Med), 5, 10, 50ng/ml IL-18 with or without 1 µg/ml inactivated influenza virus H3N2. Responses were measured as the percentage of NK cells expressing surface CD107a **(A)**, surface CD25 **(B)**, or intracellular IFN-γ **(C)** *n* = 7-8, data from a single experiment. Box plot whiskers show the 5-95th percentile range. Data were analysed with paired Wilcoxon signed rank tests. ****$p \leq 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

Furthermore, as previously reported for H1N1 virus [2], incubation of NK cells with H3N2 virus and plasma containing anti-H3N2 antibodies induced significant degranulation (CD107a expression, Figure 47A), upregulation of CD25 (Figure 47B), and production of IFN-γ (Figure 47C). However, as little as 5ng/ml IL-18 in combination with H3N2 and anti-H3N2 was sufficient to markedly augment CD107a, CD25 and IFN-γ expression; the effects of the combination of IL-18 plus H3N2/anti-H3N2 were additive for CD107a expression, but synergistic for CD25 and IFN-γ (Figure 47A-C, tests for interaction, 5ng/ml IL-18 and H3N2 for CD107a $p = 0.269$; CD25 $p = 0.002$; and IFN-γ $p = 0.038$).

Conversely, in another experiment CD4+ T cells appear to have no effect on the degranulation response to CD16 crosslinking, but contribute substantially to CD25 upregulation and IFN-γ production (Figure 48). This is consistent with the data presented in Chapter 4 showing that IL-2 blocking does not affect CD107a responses, but extends our understanding of the antigen-antibody-driven NK cell activation to suggest this pathway may be entirely CD4+ T cell independent.

In summary, therefore, IL-18 synergises with CD16-mediated signals to augment NK cell ADCC activity suggesting that IL-18 may play an important role in driving NK cell cytotoxicity as well as cytokine production.

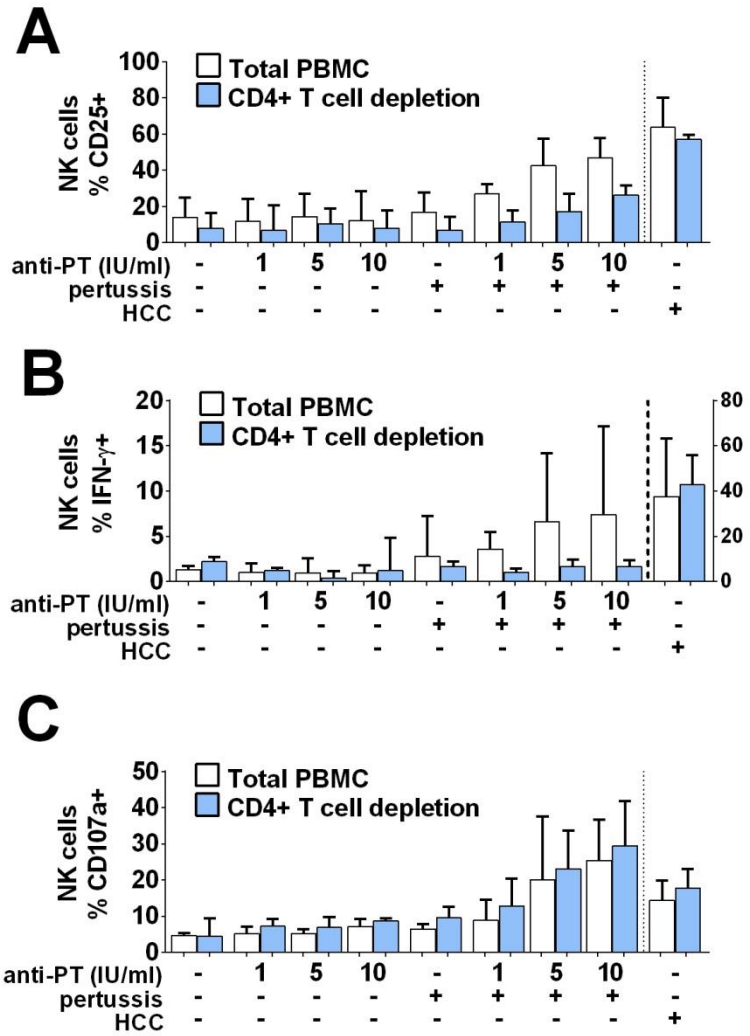

Figure 48. Degranulation responses following CD16 crosslinking are CD4+ T cell-independent. Total PBMC or PBMC depleted of CD4+ cells were stimulated for 18 hours with medium alone, 1, 5, or 10 IU/ml pertussis antiserum, and/or 1 IU/ml killed whole cell pertussis. A high concentration of cytokines (HCC: 5ng/ml IL-12, 50ng/ml IL-18) was used as a positive control. Total NK cell responses were measured in terms of upregulation of CD25 **(A)**, intracellular IFN-γ **(B)**, or CD107a **(C)**. Note that responses to HCC in **(B)** are plotted on the right-hand y axis. Bars represent medians and lines denote interquartile ranges. *n* = 4, data from a single experiment but similar results were obtained with a separate titration in total PBMC only.

5.4 Discussion

The capacity of NK cells to be activated, within minutes or hours, by very low concentrations of innate cytokines is integral to their role as early responders during infection. While pathogens may, ultimately, be cleared by components of the adaptive immune system, NK cells and other innate leucocytes are critical for initial containment of infection and orchestration of the subsequent adaptive response [31].

The role of exogenous cytokines in driving NK cell responses has been studied extensively in different contexts, including infection and cancer immunotherapy. However, these *in vitro* experiments have, almost exclusively, been carried out with purified NK cells stimulated with very high concentrations of cytokines that do not reflect the *in vivo* response, may over-ride natural homeostatic mechanisms that regulate the extent and duration of NK cell activation, and ignore interactions with other immune cells and with components of the adaptive immune system. Importantly, few studies have evaluated combinations of more than two cytokines and none have carefully titrated cytokine concentrations within these combinations. Thus, although we have abundant information about which cytokines and other signals can (under certain conditions) activate NK cells we have a much less clear picture of which signals, and which combinations of signals, most efficiently activate NK cells in physiologically relevant conditions.

In an attempt to conduct a more physiologically relevant analysis of NK cell-cytokine interactions, Asia and I have conducted a systematic analysis of the roles of different cytokines and cytokine combinations in NK cell activation and their interaction with adaptive immune responses. We have demonstrated that NK cells can respond, within hours, to concentrations of cytokines that are orders of magnitude lower than previously appreciated and that NK cells can integrate signals, synergistically, from multiple cytokine receptors and CD16, enabling

them to respond quickly and effectively to extremely low concentrations of pro-inflammatory stimuli.

Our study differs from published work not only in the very low concentrations of stimuli used, and the use of multiple cytokines in combination, but also in that we have analysed NK cell responses within whole PBMCs (containing other lymphocyte populations as well as monocytes, macrophages and dendritic cells) rather than using purified NK cells. Whilst the use of purified NK cells removes extraneous signals and allows the effects of precisely controlled cytokine concentrations to be evaluated, this approach negates the potential role of secondary cytokine responses and cell-cell contact-mediated signals which may potentiate the effect of the test stimulus [32]. I fully accept that, in our assays, exogenous cytokines might induce other cells in the PBMC population to express accessory molecules or produce cytokines that augment NK cell responses, and indeed this may explain the rather limited contribution of exogenous IL-12 in our assays, but I would argue that this better reflects the *in vivo* situation and the true potential of NK cells.

Removing accessory cells from culture abrogates many important cell-cell signals; for example, contact between NK cells and dendritic cells is required for optimal presentation of IL-15 via IL-15-IL-15Rα complexes, reducing the minimally effective IL-15 concentration from the nanomolar to picomolar range [33,34]. Similarly, interactions between NK cells and macrophages mediated, for example, by NKG2D and ICAM-LFA-1, are required for NK cell activation in numerous infection models [11,32,35,36]. In the absence of these co-stimulatory signals, responses of isolated NK cells to rather high concentrations of exogenous cytokines represent only a very incomplete picture of their true potential. Moreover, NK cell isolation may, in itself, introduce artefacts. Negative selection with agonistic anti-CD3 antibodies risks leaving behind a residue of highly activated, cytokine-producing T cells which may confound analysis of responses to other cytokines, and positive selection of NK cells requires crosslinking

of surface receptors which may positively or negatively affect the subsequent NK cell response. On balance therefore, I suggest that the experiments reported here better reflect physiological conditions during early infection and provide novel insights into NK cell activation in this context.

Our data point to IL-18 as key component of the initial inflammatory response that 'primes' NK cells to respond to other cytokines and to CD16-mediated signals. In accordance with published data [14] we show that 50ng/ml IL-18 is sufficient to induce CD25 expression on >40% of NK cells within 18 hours, however we extend these data to reveal significant upregulation of CD25 within 6 hours at IL-18 concentrations as low as 10ng/ml. Rapid IL-18-induced upregulation of CD25 explains the synergistic interaction we observed between IL-18 and IL-2; this has been reported previously but only at IL-18 concentrations that are 100-fold [37] higher than the concentration used here. Moreover, our data extend these findings to demonstrate that synergism between IL-18 and IL-2 enhances IFN-γ production (irrespective of the presence of IL-12 or IL-15) and we confirmed the importance of IL-18 in enhancing ADCC responses to influenza virus via naturally occurring anti-H3N2 antibodies in normal human plasma. Taken together, these data place IL-18 at the interface of innate and adaptive activation of NK cells.

Our data reveal that γ_c cytokines are key partners of IL-18 in this process. IL-2, IL-15 and IL-21 all signal via the common γ chain receptor, CD132, whilst receptors for IL-2 and IL-15 also share the β subunit, CD122 [20,38,39]. Although IL-2 and IL-15 are often considered functionally interchangeable as a consequence of their shared STAT5 signalling pathway (reviewed in [39]), we find that resting NK cells are much more sensitive to IL-15 than to IL-2. This likely reflects the very low levels of expression of the high affinity IL-2Rα (CD25) on resting NK cells; once CD25 is upregulated, IL-2 is not only an extremely potent inducer of NK cell IFN-γ production but also further upregulates its own receptor in an autocrine, positive feedback

loop. Importantly, however, although IL-15 and IL-18 both individually and synergistically upregulate CD25 expression, and IL-18 subsequently synergises with IL-2 to increase IFN-γ production, there is no such synergy between IL-15 and IL-2. Rather, adding IL-2 to any cytokine cocktail containing IL-15 reduces CD25 expression and has little if any beneficial effect on IFN-γ production. This is consistent with evidence that IL-2 reduces transcription of *IL15RA* [21], thereby limiting further signalling by IL-15, and may represent an important homeostatic mechanism to constrain innately driven NK cell responses once an effective adaptive immune response is underway.

Sequential activation through shared receptor components, initially by an innate cytokine (IL-15) and thence by an adaptive cytokine (IL-2), would provide a mechanism of NK cell activation that is both efficient and self-limiting, and indeed there is evidence suggesting that sequential activation of human NK cells with IL-15 and then with IL-2 potentiates STAT5 expression [14,20]. Moreover, downregulation of IL-15Rα expression by IL-2 [21] may reduce competition between IL-15Rα and IL-2Rα for the β and γ chains, facilitating formation of high affinity IL-2R and thus potentiating IL-2 signaling. Competition between IL-2 and IL-15 may extend to the shared STAT5 pathway and may explain the lack of competition with IL-21 which, although sharing the common γ chain receptor, signals via STAT3 [39].

We show that IL-18 also synergises with IgG/CD16, with as little as 10ng/ml IL-18 enhancing NK cell degranulation and IFN-γ production within 6 hours. IL-18 has previously been reported to enhance IFN-γ production and ADCC following CD16 ligation, albeit only at a 10-fold higher IL-18 concentration than employed here [40]. The discrepancy between these two studies may be explained by the much longer (overnight) incubation times used by Srivastava *et al* [40] as we found that IL-18/anti-CD16-induced responses were well past their peak after 18 hours, possibly as a result of the very rapid downregulation of CD16 expression by IL-18 that we also observed. Although downregulation of CD16 by IL-18 has been observed previously [18,40] it

was reported only at high IL-18 concentration (100ng/ml or 1μg/ml) after periods of 1-5 days and the effect was not fully quantified. The speed with which CD16 is downregulated, the very low concentrations of IL-18 required to induce it, and the synergy with CD16 crosslinking have not previously been appreciated and may, again, represent a homeostatic control mechanism to prevent excessive NK cell cytolytic activity and associated tissue damage.

Taken together, our data lead us to propose the following model of early NK cell activation (Figure 49). At the start of a primary infection, the initial colonising pathogens induce the release of constitutively expressed IL-15, activation of constitutively expressed IL-18 precursor and secretion of bioactive IL-18, and transcription and translation of *IL15* and *IL18* by dendritic cells and macrophages (reviewed in [41-43]). The synergistic interaction of IL-15 and IL-18 rapidly induces NK cells to produce IFN-γ (within 6 hours); this response may be further augmented by IL-12 and is sustained (for at least 18 hours) by a positive feedback loop in which IL-18 sustains expression of IL-18Rα. At the same time, induced expression of CD25 allows formation of the high affinity IL-2R, priming the NK cells to take part in T cell-mediated adaptive immune responses. Later in infection, or during re-infection, after the differentiation of antigen-specific T helper cells and production of antibodies, IL-18 synergises with IL-2 and with antibody-antigen complexes to enhance ADCC and IFN-γ production. The immediate availability of IgG antibodies allows ADCC reactions to occur within 5 hours [28], and subsequent rapid downregulation of CD16 by IL-18 and/or CD16 crosslinking brings the reaction to a close, thereby preventing immune pathology. T cell help is not required for this antibody-driven NK cell activation. As IL-2 signalling commences, downregulation of IL-15Rα and/or competition for β and γ chain receptor components inhibits further IL-15 signalling. IL-2Rα expression is now sustained by IL-18 and IL-2, further enhancing NK cell sensitivity to IL-2 and, in synergy with IL-12, maximising IFN-γ production [14].

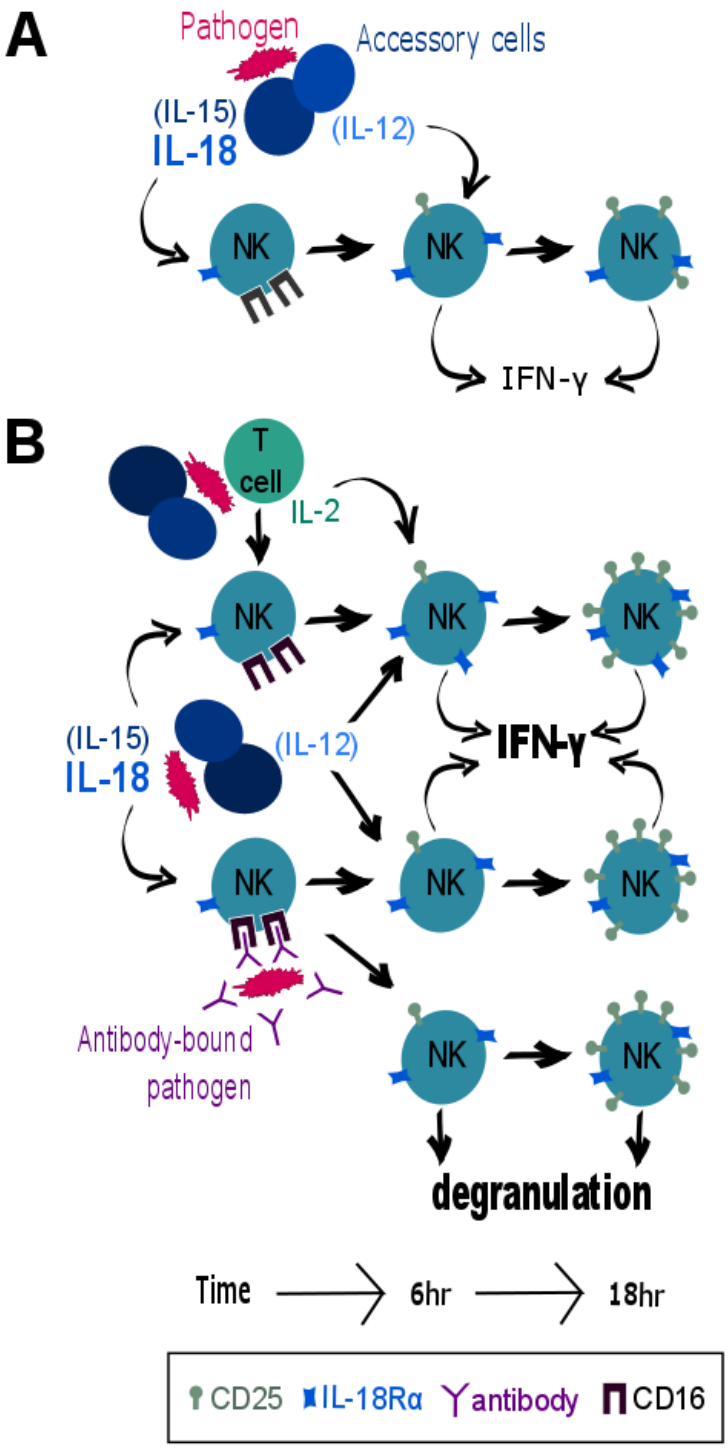

Figure 49. Rapid activation of NK cells during primary and secondary immune responses. The schematic shows our proposed model of NK cell activation. (**A**) During a primary infection, accessory cells produce IL-18 and IL-15 upon contact with the pathogen, which, within 6 hours, drives upregulation of IL-18Rα, downregulation of CD16, and low levels of CD25 expression and IFN-γ production. Subsequently, IL-18Rα expression continues to increase to at least 18 hours and CD25 expression is significantly enhanced. IL-15 and IL-18 may also synergise with IL-12 to drive a more sustained IFN-γ response. (**B**) During a secondary infection with the same pathogen, IL-2 from pathogen-specific T cells signals via CD25 and synergises with IL-18 to drive substantial IFN-γ secretion; IL-18Rα expression is further enhanced, creating a positive feedback loop. Additionally, antigen-antibody complexes crosslink CD16; this synergises with IL-18 to drive both the ADCC pathway (degranulation) and further IFN-γ production after 6 and 18 hours.

We have therefore shown that IL-18 synergises with components of both the innate (IL-15, IL-12) and the adaptive immune response (IL-2, antibody) to very rapidly induce antimicrobial NK cell responses (IFN-γ and ADCC). The extremely low concentrations of cytokines that are required for this process, and the speed with which it happens, identify IL-18 as a key 'first responder' at the intersection of innate and adaptive immune responses to infection. We have shown that IL-18Rα expression is lower across all CD56/CD57-defined subsets in HCMV+ donors, and that NK cell responses to high concentrations of IL-12/IL-18 were reduced in HCMV+ donors (Chapters 4, [2]). This strongly suggests that the fine-tuning of cytokine synergies and sensitivities, particularly those involving IL-18, could play a role in the impaired vaccine responses in HCMV+ individuals. More specifically, although NK cells in HCMV+ individuals may only have slightly lower IL-18Rα expression than HCMV-, this may ultimately lead to significant differences in cytokine and IgG responsiveness due to the ability of IL-18 to drive CD25 (high affinity IL-2R), IL-18Rα, maintain IL-15Rα, and synergise functionally with IgG-CD16 crosslinking (Chapter 4).

The working hypothesis has thus been that changes to the NK cell population are solely responsible for mediating impairment of the NK cell contribution to vaccine recall responses in HCMV+ donors, as simulated by *in vitro* stimulations with both pertussis and H1N1 (Chapter 4, [2]), although the mechanisms for this remain uncertain. This conclusion was supported by ELISPOT data demonstrating that there was no difference in IL-2 production between HCMV- and HCMV+ individuals in response to pertussis (Chapter 4, [2]). However, IL-2 is not the only cytokine involved in driving NK cell responses, and we have subsequently shown in this chapter that IL-18 is a key mediator of the synergy between innate and adaptive pathways of NK activation. Taken together, the data from Chapters 3-5 strongly suggest that sensitivity to IL-18 signalling likely plays a key role in vaccine responsiveness and heterogeneity between donors, and the impact of HCMV infection.

Conversely, it is feasible that innate cytokine availability rather than responsiveness may play more of a role if cytokine production by accessory cells in response to vaccine stimulation is not comparable between HCMV- and HCMV+ individuals. We have not explored the possibility that reduced NK cell activation in HCMV+ donors could be partially attributable to poor pro-inflammatory cytokine production. While accessory cell cytokine production of IL-12/ IL-18 in response to vaccine antigens is unlikely to explain the differences in NK cell activation between HCMV- and HCMV+ donors, limited production of these two cytokines could clearly be detrimental. Interestingly, comparison of HCMV- and HCMV+ individuals in response to H1N1 as part of an influenza vaccine intervention study (Goodier, Rodríguez-Galán, Lusa, Nielsen *et al*, manuscript accepted) did indicate a modest reduction in the median concentration of IFN-α in HCMV+ individuals (HCMV-, 401pg/ml; HCMV+, 194.3pg/ml). Furthermore, blocking IFN-αβR impaired CD25 upregulation on CD56dim NK cells in response to H1N1. It is therefore feasible that the extent to which IFN-α synergises with other cytokines in the context of antigen responses is underappreciated by the interpretation of data in this chapter.

Additionally, it is possible that differences in other cytokines or contact-dependent signals from accessory cells could also drive significantly different NK cell responses in HCMV- and HCMV+ individuals; as discussed more broadly in Chapter 1, we know that NK cell activation by dendritic cells or monocytes can occur through a variety of mechanisms (reviewed in [11,32]). Indeed, there is evidence to suggest that HCMV can affect accessory cells, which should not be surprising given that the HCMV life cycle makes extensive use of myeloid cells, both for initial infection but also for latency and subsequent viral replication [44]. Of interest in the context of infection, *ex vivo* data from two-year old children has suggested an association between reduced frequencies of CD14+CD16+ monocytes (potent secretors of IL-12) in Epstein Barr virus (EBV)/ HCMV-seropositive children and decreased NK cell responses to peptidoglycan and IL-15 [45].

To note, while our flow cytometry experiments did not include staining panels with antibodies against both CD14 and CD16, thus precluding comparison of CD14++CD16- and CD14+CD16+ monocytes between HCMV- and HCMV+ donors, I performed a crude analysis comparing proportions of CD14+ and CD14++ cells from 52 donors (HCMV-, n = 33; HCMV+, n = 19)[1]. There were no significant differences in the percentage of either subset between HCMV- and HCMV+, including when I statistically adjusted for EBV serostatus in STATA (as determined by ELISA, see Chapter 4).

This specific study with the two-year old children is difficult to interpret conclusively, in terms of the impact of HCMV, due to the confounding EBV infection status. Nonetheless, there was a significant trend to decreasing IFN-γ production by NK cells in response to IL-15 with peptidoglycan from EBV-HCMV- donors to EBV+HCMV- to EBV+HCMV+, indicating an impact of HCMV independent of EBV serostatus. These results are consistent with many studies demonstrating the impact of HCMV infection on accessory cell activity, differentiation and longevity. For example, HCMV infection of monocytes also blocks apoptosis to allow continuation of the viral life cycle and prolonged circulation of HCMV-infected monocytes [46]. Furthermore, to our knowledge, HCMV remains the only pathogen known to directly induce monocyte differentiation into macrophages, which is essential to support viral replication ([47], reviewed in [48]). It has also been shown that monocyte-derived dendritic cells release less pro-inflammatory cytokines in response to lipopolysaccharide (LPS), including IL-12, when infected with HCMV [49]. In contrast, however, transcriptome analysis suggests that HCMV infection of monocytes skews development towards the pro-inflammatory macrophage phenotype (M1, as opposed to the M2 anti-inflammatory macrophage) [50].

While further work is needed to confirm these apparently contradictory results, it is very possible that HCMV may have different effects on different cell types at various points in

[1] CD14 *ex vivo* staining performed by Martin Goodier.

infection or the viral life cycle. Given the central role of cytokine stimulation (particularly IL-18) in NK cell responses to vaccine antigens, a better appreciation of HCMV-driven changes to accessory cells would improve our understanding of the impact of HCMV infection on NK cell contributions to adaptive immunity.

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Chapter 6

Discussion

6.1 Summary and significance of findings

Taken together, the data presented in this thesis further our understanding of the capacity of NK cells to act as effectors of adaptive immune responses and demonstrate that HCMV infection is a significant confounding factor for analysis of NK cell phenotype or function. Specifically within the context of recall responses to vaccine antigens, I have shown in **Chapter 3** that decreased IFN-γ production by NK cells in response to diphtheria toxoid, tetanus toxoid or killed whole cell pertussis is associated with acquisition of CD57 in a step-wise manner from CD56dimCD57- to CD56dimCD57int to CD56dimCD57+. Consistent with the central role of pro-inflammatory cytokines IL-12 and IL-18 in driving this response, I demonstrated that surface expression of IL-12Rβ2 and IL-18Rα was lowest on these CD56dimCD57+ NK cells, suggesting that decreased sensitivity to cytokine stimulation during vaccine antigen co-culture may be contributing to these impaired NK responses. Conversely, the degranulation response (a proxy for cytotoxic activation) was maintained across all subsets.

Given that the maturation status of the NK cells was highly relevant to their ability to contribute to adaptive responses, I hypothesised that HCMV infection — which drives the expansion of a mature CD56dimCD57+NKG2C+ NK cell subset — would be associated with poorer responses to vaccine antigens. I therefore next investigated, in **Chapter 4**, the impact of HCMV-driven functional differentiation on NK activation to vaccine antigens. Here I found that, compared to HCMV- individuals, HCMV+ individuals had lower NK cell responses to killed whole cell pertussis or inactivated whole virus H1N1, interestingly both in terms of IFN-γ production and degranulation. Furthermore, differences persisted even when controlling for age and sex.

These total NK cell population data supported my hypothesis, but when I proceeded to compare the responses of specific CD57/NKG2C-defined NK cell subsets, I discovered that the impaired responses to vaccine antigens in HCMV+ individuals were not restricted to the

mature CD56dimCD57+NKG2C+ population. This suggested that HCMV infection was driving intrinsic NK cell changes across the entire NK cell population, in addition to the specific expansion of the CD56dimCD57+NKG2C+ cells that is strongly linked with HCMV seropositivity and reduced sensitivity to exogenous cytokine stimulation. Interestingly, HCMV+ individuals had lower *ex vivo* surface expression of IL-18Rα than HCMV- individuals, across all NK cell subsets.

Although there was no significant correlation between *ex vivo* cytokine receptor expression and the *in vitro* functional read-outs, I still suspected that the differences between HCMV- and HCMV+ individuals were likely linked to cytokine sensitivity or downstream signalling. In **Chapter 5**, I thus decided to more thoroughly investigate the early events in NK cell activation with combinations of very low concentrations of innate and adaptive cytokines. In conjunction with Asia-Sophia Wolf, I demonstrated a central role for IL-18 alongside common γ chain family cytokines, such as IL-15 and IL-2, in inducing production of IFN-γ as well as degranulation responses. I also found that IL-18 synergised with CD16 crosslinking, simulating a putative *in vivo* synergy between innate cytokines and antibody-antigen complexes driving antibody-dependent cellular cytotoxicity (ADCC) during a recall response. Given the decreased IL-18Rα expression on HCMV+ individuals, this cytokine work supports a model where HCMV infection influences cytokine sensitivity or downstream signalling, resulting in impaired responses to pathogens or vaccine antigens that depend on cytokine stimulation for full activation of NK cell responses.

Finally, it is important to emphasise that perhaps one of the most important contributions of this thesis work is the demonstration that HCMV is a major confounder of any study of human NK cell phenotype or function, even in healthy adults. While there is certainly a genetic component to the variation between NK cell responses, the differences between HCMV- and HCMV+ donors clearly show that much NK cell variation is attributable to environmental

factors, including HCMV infection. Data interpretation in future studies would therefore be substantially strengthened by controlling for the HCMV serostatus of study blood donors.

6.2 Opportunities for future work

6.2.1 FcεRIγ-deficient NK cells and gene expression analyses

One of the most striking findings of this thesis is that the functional impact of HCMV infection on NK cell responses to vaccine antigens is not restricted to the CD56dimCD57+NKG2C+ subset. This therefore raises mechanistic questions, and I would hypothesise that HCMV-driven changes in gene expression across all NK cell subsets are implicated in this impaired activation. One possibility is that there are deficiencies in key proteins involved in downstream signalling pathways shared between CD16 crosslinking (i.e. ADCC) and cytokine-driven activation. For example, does HCMV infection affect expression of transcription factors or other key molecules related to IL-18 signalling?

Recent investigations have identified an interesting subset of NK cells that lack expression of FcεRIγ, an adaptor protein associated with Fc receptors including CD16. HCMV+ individuals have an increased proportion of FcεRIγ-deficient NK cells, so-called 'g- NK cells', which is not entirely restricted to the CD56dimCD57+NKG2C+ subset [1,2]. These g- NK cells do not respond as well to target cells, as measured by degranulation or IFN-γ production, though there are no data available for exogenous cytokine stimulation. Further investigation of FcεRIγ deficiencies alongside functional NK cell data is therefore warranted, as it is possible that FcεRIγ-deficient NK cells in each of the NK cell subsets are responsible for the reduced responsiveness in HCMV+ individuals to vaccine antigens.

Gene expression profiling by Lee *et al* [3], comparing 'memory-like' NK cells that are deficient in FcεRIγ expression (g- NK cells) and associated with HCMV infection, to NK cells in the same donor with 'normal' FcεRIγ expression, alludes to a possible mechanism for this decreased

responsiveness in g- NK cells. Proxy markers were used to sort NK cells from two donors into populations enriched or not for g- NK cells, and microarray analysis revealed 407 differentially expressed transcripts between these two populations. The g- NK cells had 189 upregulated and 218 downregulated genes as compared to NK cells with normal FcεRIγ expression [3]. Lee *et al* were interested in understanding enhanced responsiveness to CD16 crosslinking in g- NK cells and thus focused on expression of genes encoding signalling molecules downstream from CD16. No differences in signalling proteins could be observed between g- and normal NK cells in one of the two donors, but SYK (a tyrosine kinase) expression was substantially decreased in g- NK cells in the other. The authors went on to screen a larger group (n = 62) and demonstrated that a SYK deficiency is strongly associated with HCMV infection ($p < 0.005$), but not with herpes simplex virus (HSV)-1 or HSV-2. These SYK deficiencies were associated with hypermethylation of a region of the SYK promoter, an epigenetic modification which is generally associated with downregulation of gene transcription.

Epigenetic studies that characterise these differences in methylation patterns may therefore provide further useful information to discriminate between NK cells from HCMV- and HCMV+ subjects. It would be particularly interesting to extend the work of Luetke-Eversloh *et al* who reported epigenetic demethylation at the *IFNG* locus in NKG2Chi NK cells in HCMV+ individuals, permitting enhanced IFN-γ production and overall changes in the global methylation profile to resemble memory CD8+ or Th1 T cells [4]. While this is consistent with robust IFN-γ responses by these cells to HCMV-infected target cells in the presence of anti-HCMV antibodies [5-7], it is not intuitively consistent with the reduced IFN-γ responses I observed in *all* CD57/NKG2C-defined subsets to vaccine antigens, suggesting that these HCMV-driven differences may be due to effects upstream of IFN-γ production.

Microarray work was also reported in the same issue of *Immunity* as Lee *et al* by Schlums *et al* [8] who compared ‘adaptive’ (CD56dimCD57+NKG2A-NKG2C+) and ‘mature’ (CD56dimCD57+

NKG2A-NKG2C-) NK cells between four donors and characterised transcriptional changes associated with HCMV seropositivity (CD57+NKG2C+) and with ageing (CD57+NKG2C-). They identified 89 genes that were upregulated and 102 that were downregulated genes in 'adaptive' NK cells compared to 'mature' NK cells. Transcripts for signalling/ transmembrane proteins (including FCER1G) and cytokine receptors (including IL12RB2, IL18RAP, IL2RB) were among those downregulated in the 'adaptive' cells. Similarly to the work with g- NK cells, the authors also detected downregulation of SYK on 'adaptive' NK cells. However, the most strongly downregulated transcription factor was ZBTB16, encoding PLZF, which was 77% lower in 'adaptive' as compared to 'mature' NK cells. The results from these two studies [3,8] remain broadly consistent with each other though, as Lee *et al* also established that essentially all g- NK cells were deficient in PLZF; this suggests the 'adaptive' NK cells described by Schlums *et al* have substantial overlap with these 'memory-like' g- NK cells of Lee *et al*.

Schlums *et al* went on to show using flow cytometry (*n* = 196) that a PLZF deficiency was strongly associated with HCMV infection and reduced IFN-γ responses to stimulation with innate cytokines (IL-12, IL-18); this is consistent with PLZF regulation of IL12RB2, IL18RAP, and also FcεRIγ [8,9]. However, Schlums *et al* concluded there was no absolute defect in cytokine production in PLZF-deficient NK cells because IFN-γ and degranulation responses were equally robust in response to PMA/ ionomycin stimulation. While this is supported by our data in Chapter 4 demonstrating that HCMV+ donors upregulate CD107a as well as HCMV- donors in response to K562 stimulation or crosslinking with anti-CD16, PMA/ ionomycin is a very potent stimulus and may compensate for any difference in activation threshold between NK cells. Again, these data are also consistent with HCMV-driven impairment of shared signalling pathways.

While neither study [3,8] specifies the HCMV serostatus of the donors selected for microarray analysis, the presence of 'adaptive' or 'memory-like' NK cell subsets indicates all donors were

almost certainly infected. Therefore rather than a true exploration of transcriptomic differences between NK cells in HCMV- and HCMV+ individuals, both papers provide a comparison of subsets within HCMV+ donors. This provides many useful clues into potential signalling deficits in the expanded subset of NK cells associated with HCMV infection, but cannot explain differences that I have observed between HCMV- and HCMV+ subjects across all CD57/NKG2C-defined subsets (Chapter 4). It would therefore be of interest to compare whole genome microarrays of unstimulated NK cells between HCMV- and HCMV+ individuals. This would provide us with an insight into differences in protein expression that can guide interpretation of our functional data presented in Chapter 4.

6.2.2 Characterising HCMV infection

In light of the heterogeneity I observed in Chapter 4 among the HCMV+ individuals, we must consider which parameters of HCMV infection may act as confounding factors. Firstly, as noted in the Discussion of that chapter, I do not know for how long the HCMV+ participants in this study have been infected; I am therefore unable to control for duration of infection, which possibly affects the degree of impact of HCMV on NK cell functional differentiation. There is also increasing speculation that the initial viral inoculum and immediate immunocompetence in controlling HCMV infection may also affect the extent of long-term immunomodulation that HCMV exerts on the immune system [10]. Indeed, while there are no strong data to link viral load or duration of infection with anti-HCMV IgG titres [11], there is evidence of a positive correlation between size of viral inoculum (mice [12]) or reactivation events (humans [13,14]) and greater expansions of HCMV-specific T cells.

It would therefore be interesting to characterise the extent of the T cell response to HCMV peptides in the HCMV+ individuals from Chapter 4, and establish whether there is any association between larger expansions in the HCMV-specific T cell populations and poorer NK cell responses to vaccine antigens. Similar to the heterogeneity in CD56dimCD57+NKG2C+ NK

cell expansions in HCMV+ individuals (Chapter 4), it is well-documented that there is extensive variation in the T cell response to HCMV. While the average proportion of CD4+ and CD8+ memory T cells that are HCMV-specific is 10%, making HCMV the most immunogenic pathogen yet described, this can range from 0.1-40% [15]. A significant correlation between the proportion of HCMV-specific T cells and NK cell degranulation or IFN-γ production to vaccine antigens would not indicate causation — I do not necessarily anticipate HCMV-specific T cells are directly influencing T or NK cell responses to pertussis or influenza — but rather act as a proxy for a link between HCMV infection kinetics and immune responses to heterologous infections. Such experiments to quantify HCMV-specific T cell responses would be relatively straightforward using HCMV-specific tetramers (e.g. [16]).

Alongside data on T cell expansions, it would also clearly be of significant value to be able to detect HCMV and obtain data on viral load itself. This is quite difficult in healthy adult donors, as the viral load is manifestly very low in latent infections. By developing a highly sensitive digital droplet PCR (ddPCR) protocol, colleagues have had some success in detecting HCMV in Gambian blood donors (predominantly children <2-years old), but these individuals will presumably have largely had more recent infections and higher viral loads (Roberts *et al*, unpublished data). However, my preliminary data (not shown) suggests HCMV can be detected in peripheral blood samples from healthy, latently-infected adult donors using this ddPCR approach, and the sensitivity of this can potentially be improved by extracting DNA from PBMC or monocytes. Indeed, several other groups have had success with PCR-based detection of latent HCMV in monocytes [17-20], total peripheral white blood cells [21], or urine [22]; reliable detection of HCMV in plasma samples may be limited to immunocompromised individuals or during acute infection (e.g. [23]).

Finally, while the number of individuals in my study with *NKG2C* deletions was too low to thoroughly analyse the effect of allele frequency on NK cell responsiveness to vaccine

antigens, our group's work with a Gambian population suggests a central role for *NKG2C* in control of HCMV infections [11]. It would therefore be very interesting to further investigate the impact of *NKG2C* genotype on NK cell contributions to adaptive immunity, and likewise to gain a better understanding of the factors that drive the profound expansion of the CD56dimCD57+NKG2C+ NK cell subset. This expansion is only observed in one third of HCMV+ individuals but is associated with even poorer NK cell responses than in HCMV+ individuals without this phenotypic skewing of the NK cell repertoire (see Chapter 4), and with global changes to methylation patterns [4]. It is very possible that expansion of this mature NK cell subset is linked to viral load, as with HCMV-specific T cells, or other parameters related to the interaction between HCMV and the immune system. For example, a recent study by Chen *et al* demonstrated that the ability of *in vitro* expanded NK cells to control HCMV viral dissemination was dependent on the amino acid sequence of UL18, which varied between different strains of HCMV [24]. Similarly, Rölle *et al* suggested that as polymoprhisms in UL40 can affect the interaction between HLA-E and NKG2A/NKG2C [25], these could also potentially impact the extent to which different HCMV strains drive CD56dimCD57+NKG2C+ expansions [26]. These data indicate that we need further information not only on the influence of host genetics on the impact of HCMV infection on NK cell functionality, but also on the role of viral genetics. The ability to detect and isolate HCMV from HCMV+ individuals will necessarily be central to this approach.

6.3 Putative positive effects of HCMV infection

The main implication of this thesis work is that HCMV infection may have a negative effect on vaccine efficacy due to impaired contributions of NK cells during secondary exposure to vaccine pathogens. Any public health impact of reduced functionality of NK cells in HCMV+ populations would require that the contribution of NK cells to adaptive responses is clinically relevant. While this remains to be demonstrated in a longitudinal study or in a clinical setting,

the indication has clearly been that any impact of HCMV infection is likely to be detrimental in the context of adaptive memory responses to vaccine antigens. While my data supports this phenomenon in healthy adults (median age 33-years), it is consistent with reported links between HCMV-accelerated immunosenescence and consequent compromised ability to respond to heterologous infections or vaccines in the elderly (see Chapter 1, reviewed in [27]).

Conversely, within the last few years, there has been an increasing recognition in the HCMV field of the potential benefits of HCMV infection in younger immunocompetent hosts [10,28]. Evidence of advantages from the mouse model, where latent murine CMV was associated with protection from *Listeria monocytogenes*, has long been used as an example of such a scenario [29]. However, a subsequent study indicated that this type of herpes-mediated protection is transient [30], and thus that the activated macrophages and increased IFN-γ serum levels implicated in bacterial resistance are likely a short-term phenomenon.

While no comparable CMV study has been conducted in humans, there has been a recent report linking higher frequencies of polyfunctional (CD107a+IFN-γ+TNFα+) CD8+ T cell responding to Staphylococcal Enterotoxin B (SEB) in young or middle-aged HCMV+ individuals, as compared to HCMV- individuals [31]. Noting that polyfunctionality of responding T cells may be better associated than frequency with protection from pathogens (e.g. [32]), the authors suggest that latent infection may thus confer an immunological advantage in immunocompetent adults, while potentially contributing to immunosenescence in old age through prolonged immune activation. This is consistent with earlier reports of enhanced CD8+ T cell proliferative responses to SEB and enhanced antibody responses to measles vaccination in HCMV+ infants [33], as discussed in Chapter 1, and also with production of IFN-γ by HCMV-specific T cells during effector responses to heterologous acute viral infections including hepatitis B virus [34]. More general positive immunomodulative effects associated with

herpesvirus infections, including protection from both infection or atopy [35], have also been proposed [28,36] and continue to be a topic of interest in the HCMV field.

Most relevant to this thesis work, there have also been influenza vaccine studies comparing HCMV- and HCMV+ individuals (published since the start of this thesis project) that have had access to much larger samples than the vaccine studies reported in Chapter 1. In contrast to the studies described earlier, two groups have detected a positive association between HCMV seropositivity and influenza vaccine responses. Firstly, Furman *et al* used a systems biology approach to evaluate the effect of HCMV on the immune systems of healthy individuals, including responsiveness to seasonal influenza vaccination. To their surprise, the authors found that, in young adults, HCMV infection was associated with enhanced antibody responses [37]. Likewise, McElhaney *et al* detected superior antibody responses in HCMV+ subjects, as measured by the ratio of post-vaccination to pre-vaccination antibody titres, in adults aged above 65 years ($p = 0.0251$, $n = 221$ [38]).

It was suggested at the 5th International Workshop on CMV and Immunosenescence that the inconsistencies between data supporting negative, neutral or positive health outcomes with HCMV infection may relate to the magnitude of the host response to HCMV itself [10]. Further work in this area, as detailed above, may help to resolve these questions. The longstanding hygiene hypothesis, which posits that pathogen exposure early in life modulates the immune system to prevent later development of allergy, clearly can apply to herpesviruses including HCMV. Understanding the impact of HCMV, and indeed the entire microbiome, is highly relevant to our understanding of the interaction of our immune systems and the environment at the highest level. Indeed, as White *et al* state in their 2012 review [28], the societal trends associated with improved hygiene in developed countries that have delayed herpesvirus acquisition could potentially reduce herpesvirus heterologous immunity and cross-protection from development of allergy in early childhood. While the public health risks from congenital

HCMV or infection in immunosuppressed individuals are patently serious, we should perhaps reconsider the role of HCMV earlier in life and in immunocompetent individuals. It would therefore be very interesting to complement the work presented in this thesis with similar vaccine studies in populations that can be studied longitudinally, in order determine how the effect of HCMV on NK cell vaccine responses is associated with age of HCMV acquisition, intensity, and strain of infection, and how this ties in with the broader picture of the effect of HCMV on public health.

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Appendices

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Functional significance of CD57 expression on human NK cells and relevance to disease

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Historically, human NK cells have been identified as $CD3^{-}CD56^{+}CD16^{\pm}$ lymphocytes. More recently it has been established that CD57 expression defines functionally discrete sub-populations of NK cells. On T cells, CD57 expression has been regarded as a marker of terminal differentiation and (perhaps wrongly) of anergy and senescence. Similarly, CD57 expression seems to identify the final stages of peripheral NK cell maturation; its expression increases with age and is associated with chronic infections, particularly human cytomegalovirus infection. However, $CD57^{+}$ NK cells are highly cytotoxic and their presence seems to be beneficial in a number of non-communicable diseases. The purpose of this article is to review our current understanding of CD57 expression as a marker of NK cell function and disease prognosis, as well as to outline areas for further research.

Keywords: CD57, NK cells, HCMV infection, ageing, chronic infection, cancer, autoimmune diseases, T cells

CD57 IS A MARKER OF NK CELL DIFFERENTIATION

CD57 was first identified on cells with natural killer activity using the mouse monoclonal antibodies Human Natural Killer-1 (HNK-1) (1) and Leu-7 (2) and was subsequently assigned the cluster of differentiation (CD) designation, CD57, at the fourth International Workshop of Human Leukocyte Antigens in 1989. HNK-1/Leu-7/CD57 was initially believed to be uniquely expressed on NK cells – and was used to define this population (1, 3) – although it was soon apparent that CD57 was expressed only on a subset of functionally distinct NK cells (4). CD57 was subsequently identified on $CD8^{+}$ T cells (5–7) as well as cells of neural crest origin (1, 8–13). Indeed, it was the neuroscience community that ultimately defined CD57 as a terminally sulfated carbohydrate epitope (glucuronic acid 3-sulfate) (14–16). In neural cells, the CD57 epitope is predominantly restricted to adhesion molecules (17) but little attention has been paid to the precise identity of the molecules expressing the CD57 epitope on NK cells and T cells, precluding a full understanding of the relationship between CD57 expression and lymphocyte function. Although one study identified the CD57 epitope on the IL-6 receptor gp130 of resting lymphocytes (18), the cells expressing CD57/gp130 were not identified and no comprehensive analysis of CD57-expressing molecules on T cells or NK cells has been reported.

While first characterized as an NK cell marker, CD57 has been most widely explored as a marker of replicative senescence on T cells (19). Under conditions of persistent immune stimulation, memory T cells convert from $CD28^{+}CD57^{-}$ to $CD28^{-}CD57^{+}$ (20); $CD57^{+}$ cells have short telomeres, low telomerase activity, low expression of cell-cycle associated genes and limited proliferative capacity (20, 21). However, $CD57^{+}CD28^{-}CD8^{+}$ T cells can proliferate given an appropriate cytokine milieu (22), their sensitivity to apoptosis is disputed (23, 24), they are highly cytotoxic (25, 26) and express natural killer receptors (27). $CD57^{+}CD8^{+}$ T cells should thus be regarded as terminally differentiated, oligoclonal populations of cytotoxic cells generated in response to chronic antigen stimulation.

In light of the T cell data it was suggested that CD57 may also be a marker of NK cells with poor proliferative capacity and, perhaps, a degree of immunosenescence (21, 23, 28). Indeed, acquisition of CD57 on NK cells – following stimulation with IL-2 or coculture with target cells – correlates with maturation of the $CD56^{dim}$ NK cell subset, with lower expression of NKp46, NKp30, NKG2D, and NKG2A, and higher expression of CD16, LIR-1, and killer cell immunoglobulin-like receptors (KIRs) (29). Similarly, in hematopoietic stem cell transplant recipients exposed to human cytomegalovirus (HCMV) infection, differentiation of $CD56^{dim}$ NK cells involves acquisition of CD57, loss of NKG2A, gain of KIRs, and changing expression of homing molecules (30). These studies, together with experiments in $Rag2^{-/-}\gamma cR^{-/-}$ mice reconstituted with human hematopoietic stem cells and treated with IL-15 (30), and the observation that fetal and newborn NK cells lack CD57 (31), indicate that $CD57^{+}$ NK cells differentiate from $CD56^{dim}CD57^{-}$ NK cells in an irreversible process with highly stable expression of CD57 likely being the final step in maturation (30, 32). This differentiation is accompanied by functional changes (29, 30): compared with $CD57^{-}$ cells, $CD57^{+}$ NK cells proliferate less well in response to IL-2 and IL-15 and produce less IFN-γ in response to IL-12 and IL-18, consistent with their lower levels of IL-12Rβ mRNA (29) and reduced surface expression of IL-2Rβ and IL-18Rα (30). On the other hand, $CD57^{+}$ NK cells retain their cytolytic potential (30) and a proportion of $CD57^{+}$ NK cells are able to produce IFN-γ after crosslinking of CD16 [Ref. (29); White et al. submitted] indicating that $CD57^{+}$ NK cells are intrinsically able to produce IFN-γ but that they may have different activation requirements.

In summary, therefore, progression from $CD56^{bright}$ to $CD56^{dim}CD57^{-}$ to $CD56^{dim}CD57^{+}$ reflects a maturation pathway for NK cells (33, 34) and rather than being a marker of anergy or

immunosenescence, acquisition of CD57 represents a shift toward a higher cytotoxic capacity, greater responsiveness to signaling via CD16 and natural cytotoxicity receptors (NCRs) and decreased responsiveness to cytokines (29, 35). The extent to which CD57 expression *per se* drives these changes in function, as opposed to being a marker for cells with altered expression of other attributes of a mature NK cell, is not entirely clear and may represent a fertile area for further research. In addition, a much better characterization is required of the cell surface molecules that express the CD57 epitope, the mechanisms by which CD57 is induced on them, and its functional consequences.

CD57 EXPRESSION AND CANCER

Both $CD8^+$ T cells and NK cells are able to kill tumor cells through mechanisms including perforin/granzyme-mediated cytolysis and TRAIL- or FAS-mediated apoptosis (36). Accumulation of $CD57^+CD8^+$ T cells is seen frequently in individuals with various forms of cancer (37) and has been associated with reduced survival in those with renal cell carcinoma (38), melanoma (39), gastric carcinoma (40), multiple myeloma (41), lymphomas, acute and chronic myeloid, and lymphocytic leukemias (42), among many other examples. CD57 expression on $CD4^+$ T cells has also been associated with Hodgkin's lymphoma (43) and chronic lymphocytic leukemia (44). This association between malignancy and expanded populations of $CD57^+$ T cells is likely explained by persistent stimulation of these cells by tumor-associated antigens in the absence of effective tumor clearance (45).

NK cells were initially identified by their ability to kill malignant cells (46–48) and a large body of clinical and experimental evidence now supports their crucial role in cancer immunosurveillance (49). Reduced MHC Class I expression (50) and *de novo* expression of stress related molecules (such as B7-H6, MICA, MICB, RAE-1, MULT1, and members of the ULBP family) in malignant cells alter the balance of inhibitory (via KIRs and NKG2-CD94 heterodimers) and activating (via NCRs and NKG2D homodimers) signals for NK cells (51), leading to their activation. High frequencies of peripheral or tumor-associated $CD57^+$ NK cells are reported in cancer patients and – in sharp contrast to what has been seen for $CD8^+$ T cells – have frequently been linked to less severe disease and better outcomes (**Table 1**). This would be consistent with enhanced tumor surveillance/cytotoxicity of the mature, $CD57^+$ NK cell subset (29); whether these associations are confounded by HCMV infection status (see below) is currently unclear. In the case of advanced gastrointestinal stromal tumors treated with the chemotherapeutic agent imatinib mesylate, NK cell secretion of IFN-γ after IL-12/IL-2 stimulation was correlated with improved long-term survival (52). Since $CD57^-$ NK cells are the major subset producing IFN-γ in response to cytokines, this suggests that a heterogeneous NK cell population comprising both $CD57^-$ and $CD57^+$ subsets may be optimal for combating neoplasia. Clearly further studies, ideally longitudinal in nature and accompanied by data on potentially confounding factors, are needed to determine the roles of different NK cell subsets in combating different types of malignancies.

CD57 EXPRESSION AND AUTOIMMUNITY

Autoimmune diseases tend to be highly antigen-specific and mediated by autoantibodies or autoreactive T cells. In general, expanded populations of autoreactive $CD57^+$ T cells are associated with more severe disease – Wegener's granulomatosis (65), pars planitis (25), multiple sclerosis (MS) (66), type I diabetes mellitus (67), Graves' disease (68), and rheumatoid arthritis (RA) (69), amongst others. This likely reflects killing of vital host cells by these highly cytotoxic lymphocytes (68), although the loss of T cells with immunosuppressive potential may also play a role (67).

Perhaps surprisingly, autoimmune disease is consistently associated with reduced frequencies or absolute numbers of circulating $CD57^+$ NK cells and/or impaired NK cell cytotoxicity (**Table 2**) (70–78), suggesting that cytotoxic $CD57^+$ NK cells may play a regulatory role, preventing or suppressing autoimmune disease. In MS, peripheral NK cells lose expression of FAS during relapse and regain it during remission (70) and FAS^+ NK cells can inhibit myelin basic protein-specific T cell IFN-γ responses (79), suggesting that NK cells may regulate autoreactive T cells. On the other hand, chronic NK cell lymphocytosis (which is associated with peripheral neuropathy, arthritis, and vasculitis) is characterized by increased absolute numbers of circulating immature NK cells with low cytotoxicity (80, 81). Similarly, NK cells have been found in the inflammatory infiltrates of psoriatic skin lesions (82), in synovial fluid of joints affected by RA (83), and in pancreatic islets of type I diabetes patients (84). NK cells in the synovial fluid of patients with RA, and those infiltrating psoriatic skin lesions, are immature $CD56^{bright}$ or $CD57^-$ and able to secrete IFN-γ and TNF (85, 86), suggesting that they may contribute to the inflammation rather than suppress it (84).

Taken together, these data are consistent with the hypothesis that immature $CD57^-$ NK cells may contribute to autoimmune inflammation and tissue damage whereas more highly differentiated, cytotoxic, $CD57^+$ NK cells may fulfill an immunoregulatory role, possibly deleting chronically activated T cells, as in viral hepatitis (103).

CD57 EXPRESSION DURING INFECTION

Chronic viral infections such as HCMV (104), human immunodeficiency virus (HIV) (105), hepatitis C virus (106), and Epstein–Barr virus (EBV) (107) infections offer some of the clearest examples of expansion of $CD57^+CD8^+$ T cells, presumably as a result of persistent antigenic stimulation, and increased proportions of $CD57^+CD8^+$ T cells have also been reported in those infected with human parvovirus (108), measles (109), pulmonary tuberculosis (92), and toxoplasmosis (93). The majority of these $CD57^+CD8^+$ T cells, at least in HCMV infection, appear to be antigen-specific and their presence is associated with a low incidence of reactivation (94, 95). Similar skewing of NK cells toward the $CD57^+$ phenotype is now reported in a variety of viral infections (**Table 2**).

Increased frequencies of $CD57^+CD16^+$ NK cells were first reported in HCMV-infected individuals by Gratama et al. (110) and have been repeatedly confirmed (99, 111, 112). Studies of hematopoietic stem cell transplantation (HSCT) have been particularly informative, allowing detailed comparison of stem cell differentiation into NK cells in HCMV-infected and uninfected transplant recipients (111, 112) with rapid and persistent expansion of $CD57^+$ NK cells that are also $NKG2C^+$, KIR^+, $CD158b^+$, and potent producers of IFN-γ after stimulation with MHC Class I-deficient target cells, only in the HCMV-infected group (111). We now know that HCMV drives expansion of $NKG2C^+$ NK cells and

Table 1 | Associations between cancer prognosis and CD57 expression by NK cells.

Cancer type	Observations	Reference
Acute lymphoblastic leukemia	Increased NK cell activity and increased numbers of $CD57^+$ and $CD16^+$ NK cells in bone marrow associated with complete remission	Sorskaar et al. (57)
Hodgkin's disease	Absence/low number of $CD57^+$ NK cells in tumor tissue (by immunohistochemistry) associated with relapse	Ortaç et al. (58)
Non-Hodgkin's lymphoma	Higher numbers of intratumoral $CD57^+$ NK cells are associated with relapse free survival in pediatric cases	Ortaç et al. (58)
Metastatic tumors in the brain	$CD57^+$ NK cells infiltrate brain metastases of various origins (lung, breast, and renal carcinomas; melanoma) but no correlation between numbers of infiltrating $CD57^+$ NK cells and apoptosis of malignant cells	Vaquero et al. (59)
Colorectal cancer	Increased $CD57^+$ NK cells in germinal centers of draining lymph nodes, but rarely in primary or metastatic lesions; $CD57^+$ NK cells may prevent establishment of tumor in lymph nodes?	Adachi et al. (60)
Bladder carcinoma	Lower frequency of $CD56^+$ and $CD57^+$ PBMC in patients with invasive and non-invasive tumors is correlated with reduced cytotoxicity against T24 bladder cancer cell line	Hermann et al. (61)
Breast carcinoma	Survival is positively correlated with the number of tumor infiltrating $CD57^+$ NK cells and with expression of CX3CL1 (a known NK cell chemoattractant) by the tumor cells	Park et al. (62)
Gastric carcinoma	$CD57^+$ NK cell infiltration associated with a lower clinical grade tumor, reduced venous invasion, fewer lymph node metastases, less lymphocytic invasion, and increased 5 year survival outcome	Ishigami et al. (63)
Oral squamous cell carcinoma	Low density of tumor infiltrating $CD57^+$ NK cells and high numbers of TNF^+ cells associated with higher clinical staging	Turkseven and Oygur (64)
Esophageal squamous cell carcinoma	Tumor infiltrating $CD57^+$ NK cells positively associated with increased survival over 80 months	Lv et al. (87)
Squamous cell lung carcinoma	Tumor infiltrating $CD57^+$ NK cells positively correlated with increased survival 2 years after surgery	Villegas et al. (88)
Pulmonary adenocarcinoma	Higher absolute numbers of tumor infiltrating $CD57^+$ NK cells correlated with tumor regression	Takanami et al. (89)
Various	Low numbers of $CD57^+$ NK cells in peripheral blood are associated with carcinomas of colon, lung, breast, and neck; no association was with melanoma or sarcoma	Balch et al. (90)

that these cells preferentially acquire CD57 (97–99, 111, 112). In HCMV-uninfected donors, there are roughly equal proportions of $CD57^+NKG2C^+$ and $CD57^-NKG2C^+$ NK cells whereas the ratio of $CD57^+NKG2C^+$ to $CD57^-NKG2C^+$ NK cells ranges from <1 to >60 in HCMV-infected donors (99); whether this variation reflects varying duration of HCMV infection is not known. HCMV reactivation after HSCT is associated with a threefold increase in the ratio of $CD57^+NKG2C^+$ to $CD57^-NKG2C^+$ NK cells within one year (111). Yet, in the absence of HCMV infection, $NKG2C^+$ NK cells are no more likely to acquire CD57 than are $NKG2C^-$ NK cells (112), suggesting that either binding of NKG2C to specific HCMV ligands or chronic viral infection *per se* drives NK cell differentiation. Importantly, $CD57^+CD16^+$ NK cells can kill HCMV-infected target cells (96) and this may be dependent upon, or enhanced by, α-HCMV antibodies (113).

While HCMV remains the clearest example of infection driving NK cell differentiation, other viral infections may cause a similar effect. For example, there is a three to fourfold expansion of the NK cell pool during acute hantavirus infection; NK cell numbers peak approximately 10 days after the onset of symptoms and remain above baseline for at least 60 days (114). This expansion is restricted to the $NKG2C^+$ NK cell subset and the majority of these cells are $CD57^+$, KIR^+ and highly responsive to MHC Class I-deficient target cells. Hantavirus-infected endothelial cells express high levels of the NKG2C ligand HLA-E and expansion of the $NKG2C^+$ NK cell subset is seen only in HCMV seropositive hantavirus patients, suggesting that hantavirus-induced HLA-E expression and/or inflammatory cytokines released during infection may drive the expansion and subsequent maturation of $NKG2C^+$ NK cells that have been induced or "primed" by HCMV infection (114). Similarly, transient expansion of the $CD57^+$ $NKG2C^+$ NK cell population during acute chikungunya virus infection is also associated with HCMV seropositivity (115).

Expansion of the $NKG2C^+CD57^+$ NK cell subset has also been reported in $HCMV^+$ individuals with chronic hepatitis B and hepatitis C infections, although the proportions of these cells did not differ markedly from previous reports in HCMV-infected but hepatitis virus-uninfected donors, leading the investigators to conclude that HCMV, rather than viral hepatitis, is the underlying driver of NK cell differentiation (97). In line with this, no

Table 2 | Associations between autoimmune diseases or infections and CD57 expression by NK cells.

	Observations	Reference
AUTOIMMUNE DISEASE		
Alopecia areata	$CD57^+$ NK cells are significantly reduced in peripheral blood of patients with multiple foci of alopecia	Imai et al. (91)
Atopic dermatitis	Reduced frequencies of $CD57^+$ NK cells in peripheral blood of patients compared to healthy controls, with greatest reduction in the most severe cases	Wehrmann et al. (126) and Matsumura (127)
Sjögren's syndrome	Decreased numbers of $CD57^+$ NK cells observed in peripheral blood of patients compared to controls	Struyf et al. (128)
IgA nephropathy	Decreased proportion of $CD57^+$ $CD16^+$ lymphocytes in the peripheral blood of patients compared to healthy controls	Antonaci et al.(129)
Psoriasis	NK cells infiltrating skin lesions – but also unaffected skin – are predominantly CD57low	Batista et al. (85)
INFECTION		
HCMV	Increased proportions of $CD57^+$ NK cells in infected individuals; CD57 expression limited to the $NKG2C^+$ subset	Gratama et al. (110), Lopez-Vergès et al. (99) and Foley et al. (111, 112)
HIV	In chronic infections, there is a loss of CD57-/dim NK cells, but the absolute number of $CD57^+$ NK cells remains constant	Hong et al. (100)
Chikungunya virus	Increased proportions of $CD57^+$ NK cells after infection in $HCMV^+$ patients	Petitdemange et al. (115)
Hantavirus	$NKG2C^+$ NK cell subset expanded during infection in $HCMV^+$ patients and the majority of these cells are $CD57^+$	Björkström et al. (114)
Hepatitis B and Hepatitis C	$NKG2C^+$ NK cell population is expanded in chronic infections, and these are predominantly $CD57^+$, but co-infection with HCMV appears to be the driver of this effect	Béziat et al. (97)
Lyme disease	Conflicting evidence on whether chronic disease leads to a reduced proportion of $CD57^+$ NK cells in peripheral blood	Stricker et al. (117), Stricker and Winger (118), and Marques et al. (119)

association was found between expansion of the $NKG2C^+CD57^+$ NK cell subset and clinical indicators of hepatitis such as viral load or liver enzyme concentrations (97).

In HIV-infected individuals, the absolute number of $CD57^+$ NK cells is stable and comparable to HIV-negative individuals but the ratio of $CD57^+$ to $CD57^-$ NK cells is higher than in uninfected individuals due to a gradual loss of $CD57^-$ cells (which are highly dependent on monocyte and T cell-derived cytokines for their survival) (100). Unfortunately, the HCMV status of these subjects was not reported and may confound the comparison between the HIV^+ and HIV^- individuals. Indeed, in another study, the positive association between frequency of $NKG2C^+$ NK cells and HIV-1 infection disappears when adjusted for HCMV status (101). Nonetheless, it is also the case that the frequency of $NKG2C^+(CD57^+)$ NK cells is higher in HCMV seropositive donors with HIV-1 infection than in HCMV seropositive donors without HIV-1 infection (102), suggesting either that – as for hantavirus or chikungunya virus – HIV-1 infection drives expansion of the HCMV-induced $NKG2C^+$ population or that HIV-1 infected individuals experience more frequent reactivation of HCMV which then expands the $NKG2C^+$ population. Significantly, $CD57^+$ NK cells of HIV^+ individuals retain a highly differentiated phenotype ($CD16^+KIR^+perforin^+$) but have defects in degranulation (100) suggesting that they may have reduced cytotoxic potential. Finally, although no association was seen between accumulation of $CD57^+$ NK cells and recurrence of genital herpes lesions due to herpes simplex virus 2 (HSV-2) infection (116), interpretation of this study is hindered by the lack of an HSV-2-uninfected control group.

There have been very few studies of NK cell subsets in the context of bacterial or parasitic infections. Patients with chronic Lyme Disease (*Borrelia burgdorferi*) have lower proportions of peripheral blood $CD57^+$ NK cells compared to those with acute disease and uninfected controls and this phenotype was maintained for over 10 years in one person with persistent infection (117, 118). In contrast, no significant differences in numbers of peripheral blood $CD3^-CD57^+$ cells were noted between patients with post-Lyme disease syndrome, individuals recovered from Lyme disease and healthy controls (119). The suggestion (118) that high frequencies of $CD57^+$ NK cells may be a biomarker of Lyme disease progression thus seems premature, especially given the potential impact on NK cell phenotype of HCMV and other infections.

In summary, viral infections are important drivers of NK cell differentiation with HCMV playing a primary role in selecting for $NKG2C^+CD57^+$ cells and other viruses driving their expansion and differentiation.

CD57 EXPRESSION AND AGING

Given the enormous impact of infection on NK cell maturation and differentiation, it is not surprising that NK cell populations change with age, which is a proxy for cumulative exposure to infection and other physiological insults. At birth virtually no T cells express CD57 (120) but the proportion rises with age, reaching 20–30% in young adults (20); by 80 years of age 50–60% of $CD8^+$ T cells are $CD28^-$ (and thus likely $CD57^+$) (20, 121). Similarly, with increasing age, increasing numbers of circulating NK cells are achieved by an expansion of the $CD56^{dim}$ and $CD57^+$ subsets and an absolute, as well as a proportional, decline in $CD56^{bright}$ cells (35, 53–55, 122–125). At birth, all $CD56^{dim}$ NK cells are $CD57^-$; among European adults (18–60 years of age) 25–60% of $CD56^{dim}$ NK cells are $CD57^+$ and this continues to increase slightly, but significantly, after the age of 80 years (124). Interestingly, $CD56^{dim}CD57^+$ NK cells accumulate very rapidly in an African (Gambian) population reaching adult levels (20–70%) by the age of 5 years (Goodier et al. unpublished); this may reflect very high HCMV seroprevalence rates in this age group in this community.

The increased proportion of $CD56^{dim}CD57^+$ NK cells in the elderly likely explains the maintenance of NK cell cytotoxic responses despite reduced responsiveness to cytokine stimulation [reviewed in Ref. (56)], however, the significance of these changes in terms of overall immune competence is poorly understood. The gradual loss of the $CD56^{bright}$ NK cell population, and the consequent decline in NK-derived cytokines that activate dendritic cells and monocytes, has been assumed to contribute to age-associated declines in immune competence but the potential counterbalancing effect of an increased proportion of highly cytotoxic $CD57^+$ NK cells has received little attention (123). Comprehensive studies are now needed to assess the cytokine-producing and cytotoxic function of individual NK cell subsets in response to cytokine stimulation as well as activation via CD16 and NCRs and the extent to which this changes with age and HCMV status.

CONCLUSION AND FUTURE DIRECTIONS

CD57 is a very useful marker of NK cell maturation, identifying cells with potent cytotoxic potential but decreased sensitivity to cytokines and reduced replicative potential. $CD57^+$ NK cells appear to be a stable sub-population, increasing with age and exposure to pathogens (especially, but not exclusively, HCMV) and their presence is consistently associated with better outcomes in cancer and autoimmune disease. However, the majority of clinical studies have been cross-sectional, with limited follow up and data on crucial confounding factors such as HCMV infection are typically lacking. Recent studies of HSCT (111, 112) demonstrate the power of prospective and longer term studies in beginning to assign causality in terms of NK cell phenotype, function, and disease. Nevertheless, precise understanding of the role of CD57 expression on NK cells requires a detailed dissection of the underlying biology of CD57, about which very little is known. Given that there is no evidence that CD57 is expressed on murine NK cells, this is not a simple task. Possible approaches in human NK cells might include conducting a comprehensive analysis of NK cell molecules expressing CD57, blocking CD57 in *in vitro* functional NK cell assays, or manipulating expression or enzymatic activity of B3GAT1 (the key enzyme in the biosynthesis of CD57) using RNA interference or specific inhibitors.

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IMMUNOLOGY ORIGINAL ARTICLE

Differential activation of CD57-defined natural killer cell subsets during recall responses to vaccine antigens

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Summary

Natural killer (NK) cells contribute to the effector phase of vaccine-induced adaptive immune responses, secreting cytokines and releasing cytotoxic granules. The proportion of responding NK cells varies between individuals and by vaccine, suggesting that functionally discrete subsets of NK cells with different activation requirements may be involved. Here, we have used responses to individual components of the DTP vaccine [tetanus toxoid (TT), diphtheria toxoid (DT), whole cell inactivated pertussis] to characterize the NK cell subsets involved in interleukin-2-dependent recall responses. Culture with TT, DT or pertussis induced NK cell CD25 expression and interferon-γ production in previously vaccinated individuals. Responses were the most robust against whole cell pertussis, with responses to TT being particularly low. Functional analysis of discrete NK cell subsets revealed that transition from $CD56^{bright}$ to $CD56^{dim}$ correlated with increased responsiveness to CD16 cross-linking, whereas increasing CD57 expression correlated with a loss of responsiveness to cytokines. A higher frequency of $CD56^{dim}$ $CD57^{-}$ NK cells expressed CD25 and interferon-γ following stimulation with vaccine antigen compared with $CD56^{dim}$ $CD57^{+}$ NK cells and made the largest overall contribution to this response. $CD56^{dim}$ $CD57^{int}$ NK cells represent an intermediate functional phenotype in response to vaccine-induced and receptor-mediated stimuli. These findings have implications for the ability of NK cells to contribute to the effector response after vaccination and for vaccine-induced immunity in older individuals.

Keywords: CD57; diphtheria–tetanus–pertussis vaccine; natural killer cells.

Introduction

Natural killer (NK) cells are classically regarded as a stable population of innate immune effectors that, by cytokine production or cytotoxicity, help to contain an infection or limit tumour growth until an effective adaptive response is mounted. However, numerous lines of evidence now suggest that NK cells adapt functionally after stimulation by viruses, cytokines and hapten antigens; this phenomenon has been termed 'NK memory' but may also reflect functional NK cell maturation.[1] There are several routes by which NK cell function may be enhanced during re-exposure to a pathogen. Antigen-specific memory T cells secreting interleukin-2 (IL-2) promote NK cell function and proliferation, while pathogen-specific antibodies initiate antibody-dependent cellular cytotoxicity by cross-linking CD16 or other Fc receptors for immunoglobulins.[2–6] Alternatively, cytokines released during primary infection may induce NK cells to proliferate and/or differentiate to a more highly responsive state; subsets of NK cells expressing activating receptors able to bind specific pathogen ligands may be particularly responsive (as described for the $Ly49H^{+}$ subset of mouse NK cells which bind the murine cytomegalovirus m157 protein[7]).

Abbreviations: APC, allophycocyanin; DT, diphtheria toxoid; DTP, diphtheria–tetanus–pertussis; HCC, high concentration of cytokines; HCMV, human cytomegalovirus; IFN-γ, interferon-γ; IL-2, interleukin-2; LCC, low concentration of cytokines; NK, natural killer; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin; rh, recombinant human; TT, tetanus toxoid

A number of NK cell subsets with different functional potential have now been described in humans. The least mature circulating NK cells are CD56^{bright} CD57$^-$ and are assumed to give rise to CD56^{dim} CD57$^-$ cells which, in turn, mature into CD56^{dim} CD57$^+$ cells, the latter subset increasing in frequency with increasing age.[8,9] This three-step maturation is associated with acquisition of CD16, CX3CR1, granzyme and KIR, gradual loss of proliferative capacity, reduced responsiveness to cytokines such as IL-12 and IL-18, and increasing cytotoxic function.[10,11] CD56^{dim} CD57$^+$ NK cells express lower levels of IL-18Rα[10,11] as well as lower levels of mRNA for the inducible chain of the IL-12R (IL-12Rβ2)[12] suggesting that these NK cells may respond less well than other subsets to IL-12 and IL-18. Conversely, CD56^{dim} CD57$^+$ cells express higher levels of CD16, suggesting that they may be particularly good mediators of antibody-dependent cellular cytotoxicity.[12]

The potential for NK cells to respond to exogenous cytokines is central to their ability to control infections,[4,13,14] particularly where ligands for other NK-activating receptors are lacking. Moreover, NK cells responding to CD4$^+$ T-cell-derived IL-2 have the potential to contribute to secondary immune responses, including those induced by vaccination.[3,4] We wondered, therefore, whether NK cell subsets would differ in their ability to mount 'recall' responses to vaccine antigens. To test this hypothesis, we have assessed the capacity of various NK cell subsets, defined principally by their expression of CD56 and CD57, to contribute to a recall response to the components of diphtheria–tetanus–pertussis (DTP) vaccine. We find that vaccine-induced NK cell interferon-γ (IFN-γ) and degranulation (CD107a) responses differ between NK cell subsets. Importantly, our studies reveal that CD57 expression is gained in a gradual stepwise fashion and that changes in NK cell function mirror this progressive maturation.

Materials and methods

Donors and peripheral blood mononuclear cell preparation

Volunteers were recruited from among staff and students at the London School of Hygiene and Tropical Medicine. All subjects gave fully informed, written consent and the study was approved by the London School of Hygiene and Tropical Medicine Ethics Committee. Subjects ranged in age from 21 to 73 years and all donors confirmed that they had been vaccinated against diphtheria, tetanus and pertussis in childhood. Peripheral blood mononuclear cells (PBMC) were separated by fractionation on a Ficoll–Hypaque gradient and cryopreserved in liquid nitrogen. Frozen PBMC were thawed with pre-warmed complete medium [RPMI-1640 supplemented with 100 U/ml penicillin/streptomycin and 20 mM L-glutamine (Gibco, Lifesciences, Paisley, UK) and 10% pooled human AB serum (Sigma, Poole, UK)] (at 37°), washed several times and rested for 30 min before use.

NK cell assay culture

Peripheral blood mononuclear cells (2×10^5 cells in 200 µl) were cultured in 96-well U-bottom plates in complete medium with or without low concentration of cytokines [LCC; 12·5 pg/ml recombinant human (rh) IL-12 (PeproTech, Rocky Hill, NJ) plus 10 ng/ml rhIL-18 (MBL, Woburn, MA)]; high concentration of cytokines (HCC; 5 ng/ml rhIL-12 plus 50 ng/ml rhIL-18); or 7·5 µg/ml tetanus toxoid (TT), 1 µg/ml diphtheria toxoid (DT) or 1 IU/ml whole cell pertussis (all from the National Institute for Biological Standards and Control, London, UK) for 18 hr at 37°. GolgiPlug (containing Brefeldin A, 1/1000 final concentration; BD Biosciences, Oxford, UK) and GolgiStop (containing Monensin, 1/1500 concentration; BD Biosciences) were added after 15 hr.

Receptor cross-linking

Flat-bottomed 96-well plates were coated (overnight at 4°) with 50 µl of mouse monoclonal antibody to human CD16 (final concentration of 20 µg/ml; BD Biosciences) or a cocktail of monoclonal antibodies to human NK receptors [NKG2D, NKp30, NKp46, 2B4 (all from R&D Systems, Abingdon, UK)] and CD2 (BD Biosciences) at an overall combined concentration of 20 µg/ml, i.e. 4 µg/ml each. An equivalent concentration of mouse IgG1 κ isotype control antibody (BD Biosciences) was used as a negative control. After washing (three times in sterile PBS), 2×10^5 PBMC were added to each well and incubated for 18 hr. GolgiPlug and GolgiStop were added after 15 hr. Cells were then transferred to 96-well U-bottomed plates for washing and staining.

Flow cytometry

Responses of NK cells and T cells were assessed as described previously.[15] Briefly, cells were stained with fluorophore-labelled monoclonal antibodies to cell surface molecules, fixed, permeabilized and stained for intracellular molecules using a Cytofix/Cytoperm kit (BD Biosciences). Cells were analysed by flow cytometry on an LSR II (BD Biosciences). Samples with fewer than 100 NK cells in each subset were excluded. The following reagents were used: anti-CD56-phycoerythrin (PE) -Cy7, anti-CD16-allophycocyanin (APC) -H7, anti-CD4-Pacific Blue, anti-IFN-γ-e780, anti-IFN-γ-APC, anti-CD3-V500 and anti-CD69-phycoerythrin-cyanine5 (PE-Cy5) (all BD); anti-CD8-PE-Cy5, anti-CD25-PE, anti-IL-18Rα-PE, anti-CD62L-PE-Cy5, anti-CD57-e450 and anti-IL-2-APC (all

e-Biosciences/Affimetrix, Hatfield, UK). Anti-IL-12Rβ2 monoclonal antibody was obtained from R&D Systems (Oxford, UK) and conjugated to PE-Cy5 using an Easylink PE/Cy5® Conjugation Kit (Abcam, Cambridge, UK).

Data and statistics

Unless stated to the contrary, all figures show data from at least three replicate experiments. Flow cytometry data were analysed using FLOW JO (Tree Star, Ashland, OR) and data were analysed using PRISM6 (GraphPad, San Diego, CA) software. Statistical comparisons were performed by paired analysis of variance or *t*-tests. Correlation between parameters was by bivariate regression analysis. $****P \leq 0{\cdot}0001$, $***P < 0{\cdot}001$, $**P < 0{\cdot}01$, $*P < 0{\cdot}05$.

Results

DTP vaccination induces durable vaccine antigen-driven NK cell responses

To validate DTP vaccination as a suitable model for evaluating NK cell recall responses, PBMC were incubated overnight with TT, DT or inactivated whole cell pertussis with or without low concentrations of the cytokines IL-12 and IL-18 (LCC) or, as a positive control, with a high concentration of cytokines IL-12 and IL-18 (HCC), stained for NK cell phenotypic and functional markers, and examined by flow cytometry (Fig. 1). HCC induces over 50% of $CD3^-$ $CD56^+$ NK cells to express cell surface CD25 and intracellular IFN-γ (median 19·9%, range 1·6–57·5, Fig. 1a–c) and has a significant, but much less marked, effect on CD107a expression (median 2·5%, range 0·001–9·0, Fig. 1a,d,e). By contrast, LCC alone induces a small, but significant, proportion of NK cells to express CD25 (median 6·4%, range 0·6–25·4), but few, if any, of these cells also produce IFN-γ (median 0·0%, range 0·0–1·68) or express CD107a (median 0·4%, range 0·1–2·4) on their surface (Fig. 1a).

Among PBMC stimulated with vaccine antigen alone (i.e. without LCC) there is highly significant up-regulation of both CD25 and IFN-γ by NK cells in response to pertussis (median 1·3%, range 0·0–4·6), a lesser (but still significant) response to DT (median 0·1%, range 0·0–1·3) and no significant response to TT (median 0·1%, range 0·0–1·3) (Fig. 1b). However, responses to all three antigens were significantly enhanced in the presence of LCC (pertussis: median 3·9%, range 0·9–17·6; DT: median 0·5%, range 0·0–13·5; TT: median 0·3%, range 0·0–2·13) (Fig. 1c) and were ablated in the presence of neutralizing antibody to IL-2 (data not shown). These data are fully consistent with a scenario in which a whole cell antigen such as pertussis contains ligands for Toll-like receptors[16] and so induces accessory cells to secrete cytokines such as IL-12 and IL-18, whereas purified proteins such as TT and DT do not; exogenous LCC induces expression of CD25 (and so the high-affinity IL-2R) on NK cells allowing them to respond to IL-2 from vaccine-specific $CD4^+$ T cells. By contrast, a statistically significant increase in CD107a expression on NK cells was seen in response to all three vaccine components (pertussis: median 2·2%, range 0·2–22·2; DT: median 0·5%, range 0·0–2·6; TT: median 0·5%, range 0·0–4·3) (Fig. 1d) and this was not significantly enhanced by LCC (pertussis: median 4·5%, range 0·9–20·0; DT: median 0·9%, range 0·0–3·0; TT: median 0·6%, range 0·1–2·5) (Fig. 1e).

CD57 is a stable marker of human NK cell subsets

Despite very robust NK cell responses to some of the vaccine antigens, not all NK cells responded and there is considerable heterogeneity in the magnitude of the NK cell response between donors (Fig. 1b–e). Although heterogeneity between individuals might be explained by variation in the strength of the T-cell IL-2 response that drives the NK responses,[3,17,18] this is unlikely to explain heterogeneity of responses within the NK cell population of an individual donor. We therefore considered whether within-donor variation might be the result of differences between subsets of NK cells in their intrinsic sensitivity to activation by monokines and T-cell-derived IL-2.

CD57 is a marker of highly differentiated, highly cytotoxic NK cells[12,19,20] and CD62L (L-selectin) is a marker of cells able to proliferate and secrete IFN-γ after high-dose cytokine stimulation.[21] However, to use these as markers of NK cell subsets in mixed PBMC assays, it was important to know whether they were stable phenotypic markers or whether their expression was altered after activation. To this end, expression of CD62L and CD57 were examined on PBMC after overnight stimulation with LCC or HCC, or with cross-linking antibody to the NK cell activating receptor CD16, or a cocktail of antibodies to NK cell activating receptors (NKp30, NKp46, NKG2D and CD2) (Fig. 2). Consistent with previous reports,[12] CD62L and CD57 tended to define mutually exclusive subsets of NK cells (Fig. 2a). However, although CD57 expression appeared very stable after overnight activation by cytokines or receptor cross-linking (Fig. 2b), CD62L expression was markedly reduced after activation (Fig. 2c). Given the significant activation-induced down-regulation of CD62L, subsequent functional analysis of NK subsets was based on CD57 expression but not CD62L.

CD56 and CD57 define multiple distinct NK cell subsets

Expression of CD56 and CD57 has been used to identify three subsets of NK cells. Functional analysis of these subsets suggests that NK cells differentiate from relatively immature $CD56^{bright}$ $CD57^-$ cells, which respond to

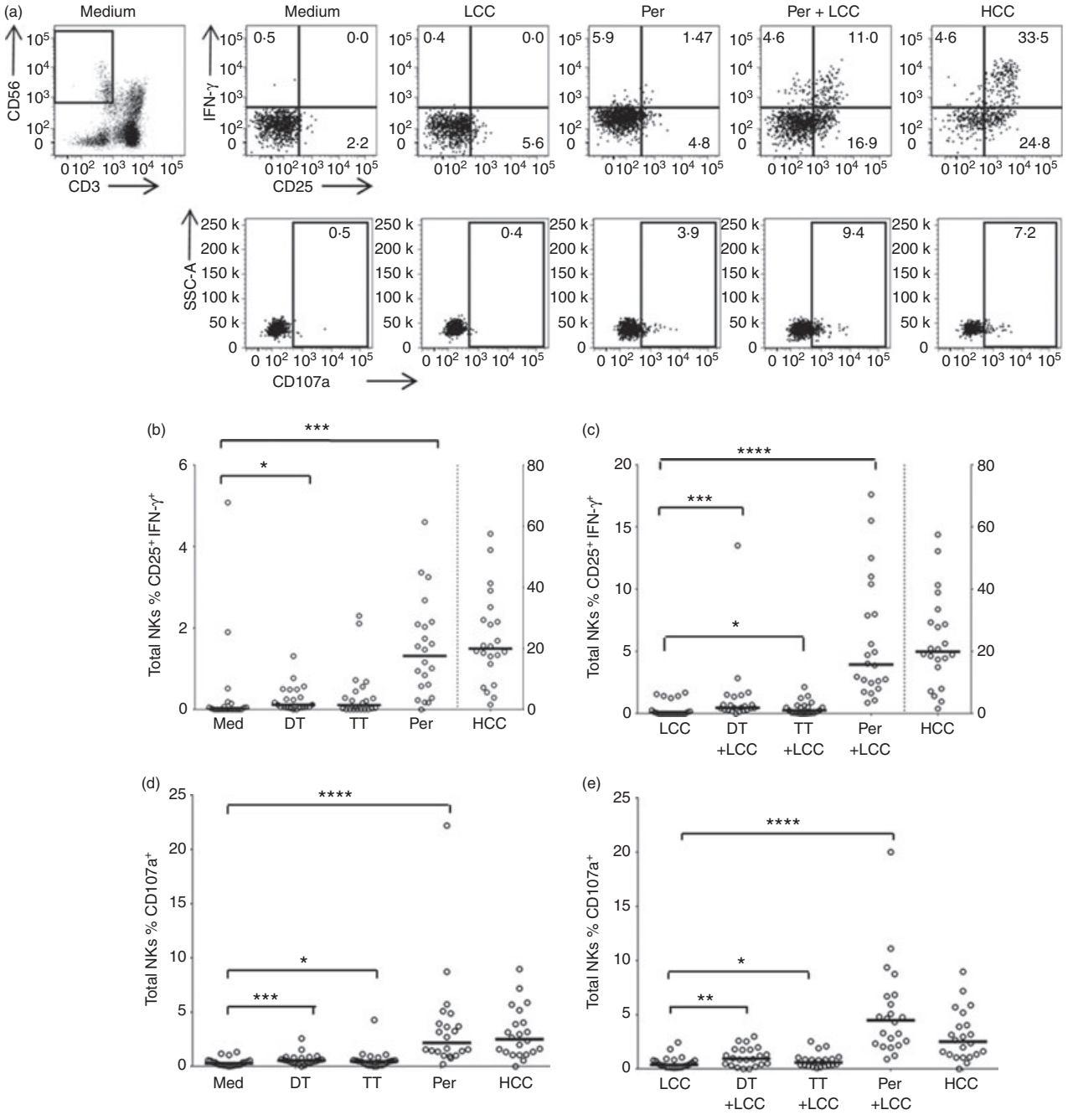

Figure 1. Natural killer (NK) cell responses to diphtheria toxoid (DT), tetanus toxoid (TT) and whole cell pertussis. Peripheral blood mononuclear cells (PBMC) from previously vaccinated donors were cultured *in vitro* for 18 hr with medium alone, low concentration of cytokines (LCC), DT, TT, pertussis (Per), DT + LCC, TT + LCC, Per + LCC, or high concentration of cytokines (HCC). (a) Representative flow cytometry plots showing gating of CD56$^+$ CD3$^-$ NK cells and expression of CD25, CD107a and interferon-γ (IFN-γ). (b, c) Percentage of NK cells co-expressing CD25$^+$ and IFN-γ^+ after stimulation in the absence (b) or presence (c) of LCC. (d, e) Percentage of NK cells expressing CD107a after stimulation in the absence (d) or presence (e) of LCC. Note: in (b) and (c) HCC data are shown on a different axis (see right hand side of plot). Each data point represents one donor, $n = 22$. Lines represent median values. Data were analysed with paired, non-parametric *t*-tests. **** $P \leq 0{\cdot}0001$, *** $P < 0{\cdot}001$, ** $P < 0{\cdot}01$, * $P < 0{\cdot}05$.

cytokine stimulation by producing IFN-γ but have limited cytotoxic potential, to CD56^{dim} CD57$^-$ cells which are also poorly cytotoxic but retain IL-12R expression and so the ability the secrete IFN-γ in response to cytokine stimulation and, eventually, to CD56^{dim} CD57$^+$ cells, which no longer respond to exogenous cytokines but are skewed towards a cytotoxic phenotype following cross-linking of CD16 or NK receptors or exposure to target

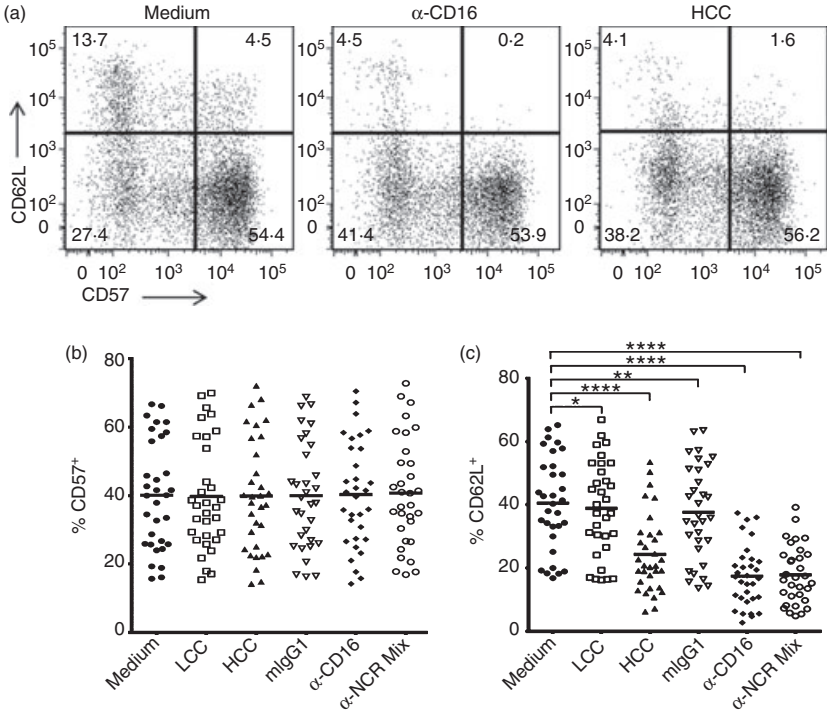

Figure 2. CD57 is a stable marker of human natural killer (NK) cell subsets. Peripheral blood mononuclear cells (PBMC) were cultured *in vitro* for 18 hr with plate-bound mouse IgG1 isotype control (mIgG1), anti-human CD16, anti-human NK cell receptor (NKR) cocktail (α–CD2, α–NKG2D, α–NKp30, α–NKp46) (all to a final concentration of 20 μg/ml), low concentration of cytokines (LCC) or high concentration of cytokines (HCC). (a) Representative flow cytometry plots showing expression of CD62L and CD57 on gated ($CD56^+$ $CD3^-$) NK cells. (b) Percentage of NK cells that were $CD57^+$ after PBMC culture under different conditions. (c) Percentage of NK cells that were $CD62L^+$ after PBMC culture under different conditions. *P*-values are derived from repeated measures analysis of variance (c). Each data point represents one donor, $n = 31$. Lines represent mean values. $^{****}P \leq 0{\cdot}0001$, $^{**}P < 0{\cdot}01$, $^{*}P < 0{\cdot}05$.

cells.[10,12,20] However, CD57 expression is not simply 'off' or 'on' but is gradually up-regulated in a stepwise fashion (Fig. 3). It was possible to identify seven distinct peaks of CD57 expression (Fig. 3b,c) with each peak accounting for ~5% to ~35% of all $CD56^{dim}$ NK cells (Fig. 3d). CD62L expression is lost as soon as cells begin to express CD57 (Fig. 3e) but CD16 expression is gradually up-regulated, with maximal CD16 expression not being reached until the third peak of CD57 expression (Fig. 3f). Most importantly, the functional remodelling of NK cells, in terms of loss of cytokine-induced up-regulation of CD25 and IFN-γ expression, is extremely gradual with complete unresponsiveness to HCC not being seen until CD57 expression reaches its maximal level (Fig. 3g,h). By contrast, little or no difference was observed in the ability of NK cells with different levels of CD57 expression to degranulate in the presence of cytokines (Fig. 3i).

These data suggest that NK cells with intermediate levels of CD57 expression ($CD57^{int}$), which represent a significant fraction (~30%) of circulating NK cells, are also intermediate in terms of their functional maturation. To formally test this hypothesis, we analysed responses of the four NK cell subsets ($CD56^{bright}$; $CD56^{dim}$ $CD57^-$; $CD56^{dim}$ $CD57^{int}$ and $CD56^{dim}$ $CD57^+$, Fig. 4a) to HCC, cross-linking of CD16 and cross-linking of NK receptors, by expression of CD25, IFN-γ or CD107a (Fig. 4b–d). As expected, high proportions of $CD56^{bright}$ cells expressed CD25, IFN-γ or CD107a in response to HCC; cross-linking of CD16 or NK cell receptors up-regulated CD25 and CD107a but not IFN-γ in this subset (Fig. 4b–d). Among $CD56^{dim}$ NK cells, CD25, CD107a and IFN-γ responses to HCC declined with increasing levels of CD57 expression with a statistically significant negative trend from $CD56^{dim}$ $CD57^-$ cells, through $CD56^{dim}$ $CD57^{int}$ cells to $CD56^{dim}$ $CD57^+$ cells (analysis of variance for all linear trends, $P \leq 0{\cdot}0001$) (Fig. 4b–d). Interestingly, although no significant differences were observed between the three $CD56^{dim}$ populations in their ability to degranulate or produce IFN-γ in response to CD16 or NK cell receptor cross-linking, the cross-linking of CD16 or NK cell receptors led to increasing levels of CD25 expression with increasing expression of CD57 (linear trend; $P \leq 0{\cdot}0001$ in both cases), suggesting that responsiveness to T-cell IL-2 may be retained in $CD57^+$ NK cells in the presence of antibodies able to induce antibody-dependent cellular cytotoxicity. In summary therefore, the transition from $CD56^{bright}$ to $CD56^{dim}$ (irrespective of CD57 expression) is coincident with a marked reduction in cytokine secretion,

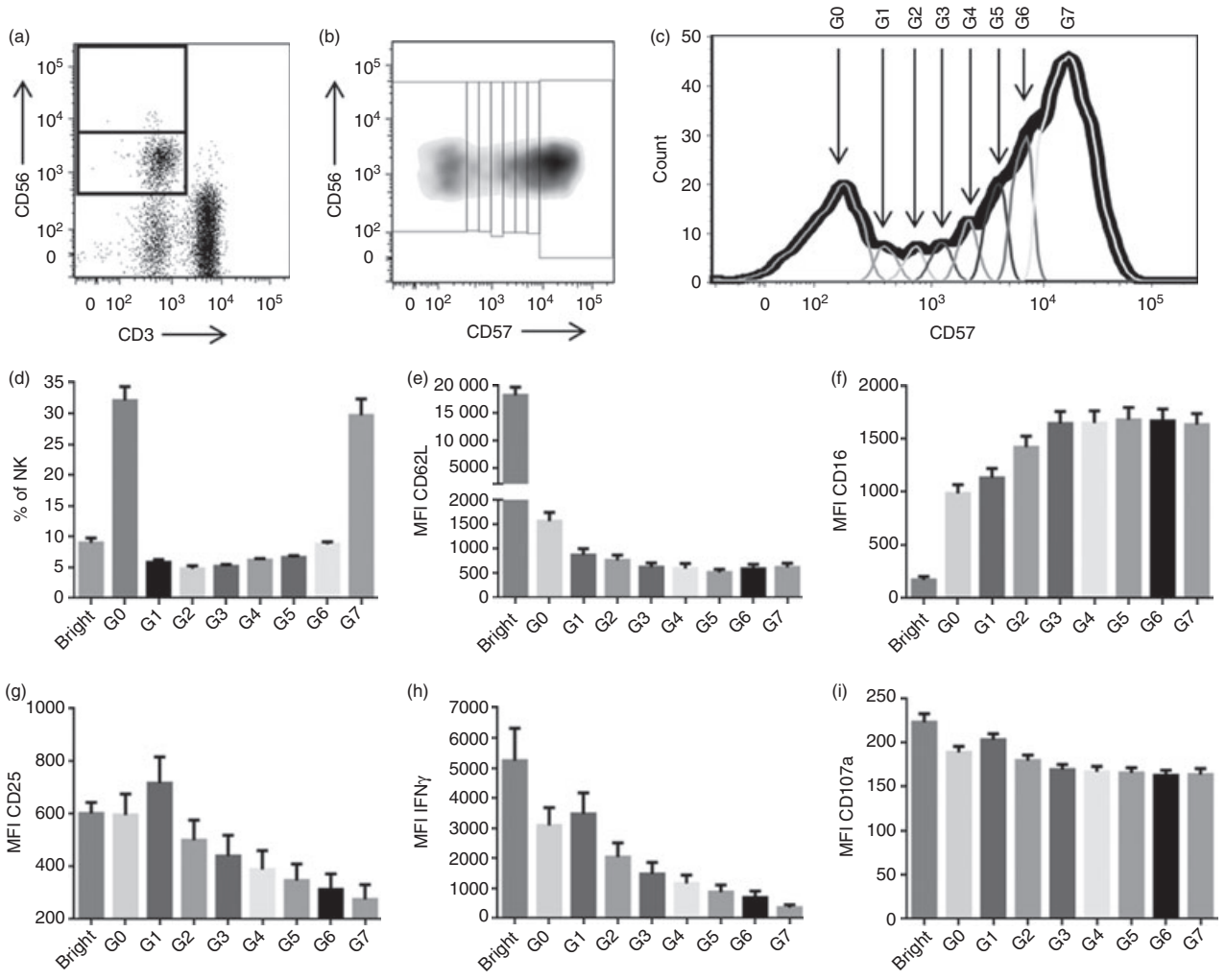

Figure 3. CD56 and CD57 define multiple distinct natural killer (NK) cell subsets. Representative flow cytometry plots showing gating of CD56^{bright} and CD56^{dim} NK cells (a) and corresponding dot plot (b) and histogram (c) showing gating of the CD56^{dim} subset into seven subpopulations based on CD57 expression. The G0 population represents CD56^{dim} CD57$^-$ cells; G1–6 are CD56^{dim} CD57^{int} cells; G7 are CD56^{dim} CD57$^+$ cells). (d–f) *Ex vivo* analysis of each subpopulation of NK cells (as defined in c) among NK cells from 32 donors: (d) mean (SEM) percentage of all NK cells which fall into each subpopulation; (e) mean (SEM) mean fluorescence intensity (MFI) of CD62L expression and (f) CD16 expression on each subpopulation. (g–i) Peripheral blood mononuclear cells from 32 donors were stimulated for 18 hr with high concentration of cytokines: mean (SEM) MFI of CD25 expression (g), interferon-γ (IFN-γ) expression (h), and CD107a expression (i) on each subpopulation. Bar charts represent means $\pm$ SEM, $n = 32$.

but no overall change in degranulation, in response to cross-linking of NK cell receptors or CD16 receptors. By contrast, increasing CD57 expression correlates with a gradual loss of responsiveness (in terms of CD25 expression, IFN-γ release and degranulation) to exogenous IL-12 + IL-18.

Vaccine-driven, cytokine-mediated NK cell IFN-γ responses are dominated by the CD56^{dim} CD57$^-$ and CD56^{dim} CD57^{int} NK cell subsets

Accessory cytokines (including IL-12 and IL-18) and T-cell-derived IL-2 are known to be essential to drive NK cell IFN-γ responses during re-stimulation with vaccine antigens.[3] Given that increasing CD57 expression correlates with loss of responsiveness to HCC, we predicted that CD56^{dim} CD57$^-$ or CD56^{dim} CD57^{int} NK cell populations would show stronger 'recall' responses to whole cell pertussis than would CD56^{dim} CD57$^+$ NK cells. To test this hypothesis, responses to pertussis (Fig. 1) were analysed for each of the four NK cell subsets defined by CD56 and CD57 expression (Fig. 5). There was a clear hierarchy of responses with a significantly higher proportion of CD56^{dim} CD57$^-$ NK cells than CD56^{dim} CD57^{int} or CD56^{dim} CD57$^+$ NK cells co-expressing CD25 and IFN-γ ($P < 0{\cdot}001$ for linear trends) (Fig. 5a). On the

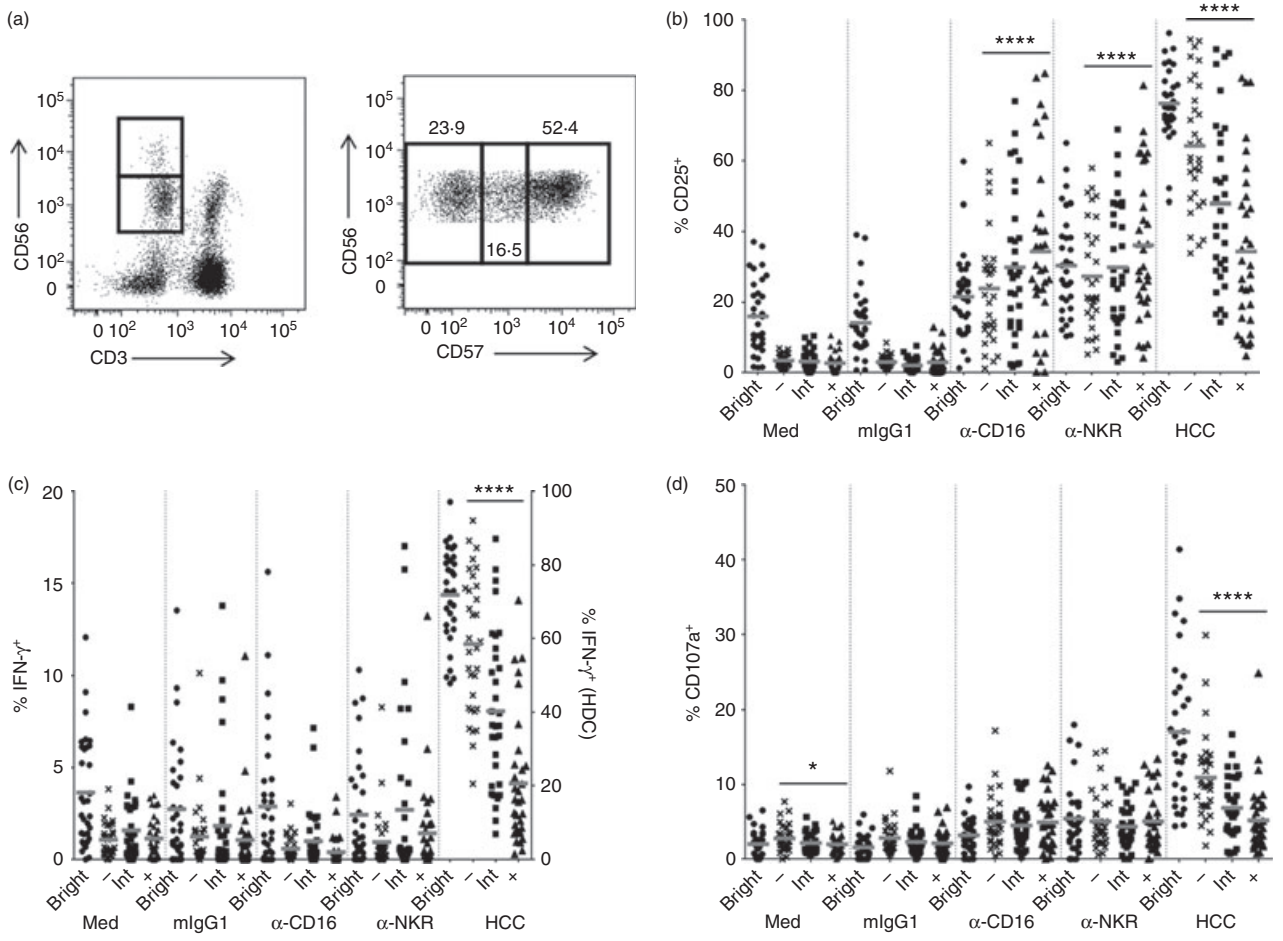

Figure 4. CD57 defines a continuum of functionally distinct natural killer (NK) cells. (a) Representative flow cytometry plots showing gating of $CD56^{bright}$ and $CD56^{dim}$ NK cells (left), and subsequent gating of the $CD56^{dim}$ subset into $CD56^{dim}$ $CD57^{-}$, $CD56^{dim}$ CD57 intermediate ($CD57^{int}$) and $CD56^{dim}$ $CD57^{+}$ populations (right). (b–d) Peripheral blood mononuclear cells from 32 donors were cultured with cross-linking antibodies to CD16 or NK cell receptors, or with an isotype control mIgG1 or high concentration of cytokines (HCC), for 18 hr. Percentage of cells in each NK subset expressing CD25 (b), interferon-γ (IFN-γ) (c) and CD107a (d). Note: in (c), HCC data are shown on a different axis (see right hand side of plot). Each data point represents one donor, $n = 33$. Lines represent mean values. $CD56^{dim}$ subsets were analysed for linear trend with a repeated measures analysis of variance. $^{****}P \leq 0{\cdot}0001$, $^{*}P < 0{\cdot}05$.

other hand, CD107a expression was similar among all three CD57-defined NK cell subsets (Fig. 5b). When considering the proportion of all NK cells belonging to each subset together with the responsiveness of each individual subset, it became evident that vaccine antigen-driven NK cell IFN-γ recall responses occur almost entirely within the $CD56^{bright}$ and $CD56^{dim}$ $CD57^{-}$ NK cell subsets with minimal contributions from the $CD56^{dim}$ $CD57^{int}$ and $CD56^{dim}$ $CD57^{+}$ subsets (Fig. 5c).

CD57 acquisition is associated with reduced expression of cytokine receptors IL-12Rβ2 and IL-18Rα

CD57 acquisition on NK cells is associated with a reduced ability to respond to accessory cytokines (Fig. 4) leading to a progressive decline in their ability to respond to vaccine-driven cellular responses by production of IFN-γ (Fig. 5a). To determine whether this is due to altered cytokine receptor expression and altered downstream signalling we assessed the resting (*ex vivo*) expression of IL-18Rα and IL-12Rβ2 (Fig. 6). The proportion of IL-12Rβ2-expressing cells was highest among the $CD56^{bright}$ NK cells with a progressive decrease in expression across the CD57-defined NK cell subsets (Fig. 6b) but IL-12Rβ2 expression density did not vary across subsets (Fig. 6c). Although IL-18Rα was expressed at a much higher frequency than IL-12Rβ2 within all NK cell subsets, the same trend was seen, with declining IL-18Rα expression, with increasing CD57 expression (Fig. 6d). In contrast to IL-12Rβ2, however, IL-18Rα mean fluorescence intensity also declined with increasing CD57 expression (Fig. 6e).

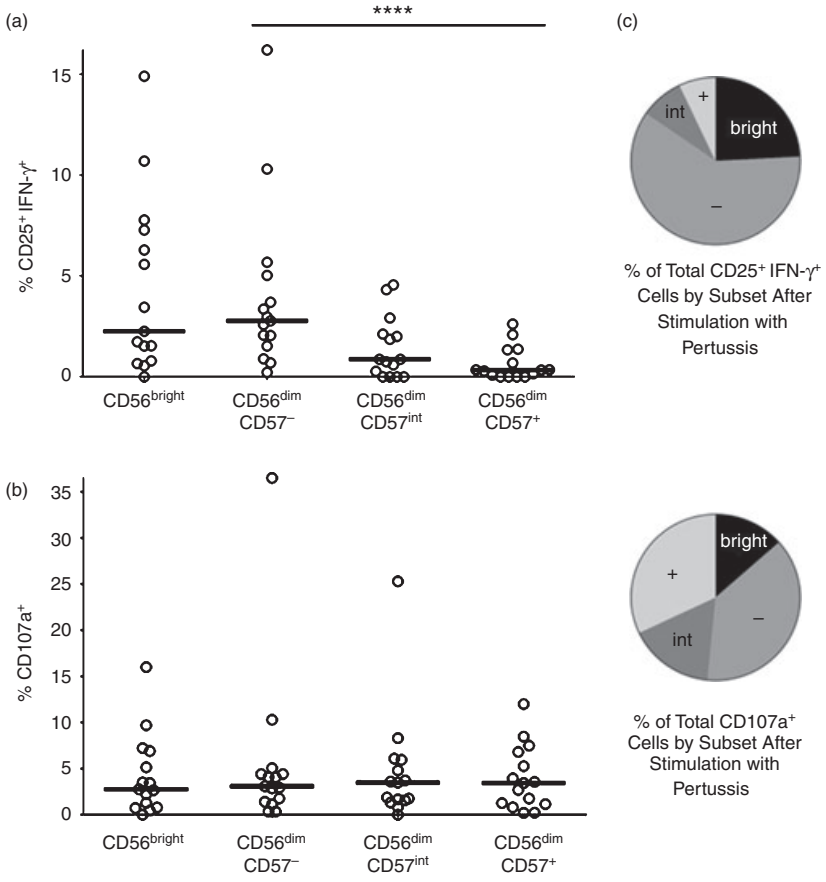

Figure 5. Natural killer (NK) cell interferon-γ (IFN-γ) responses to pertussis are dominated by the CD56^{dim} CD57^{-} and CD56^{dim} CD57^{int} NK cell subsets. Peripheral blood mononuclear cells were cultured with pertussis for 18 hr. The percentage of cells in each subset that are CD25^{+} IFN-γ^{+} (a) and/or CD107a^{+} (b) is shown. Each data point represents one donor, $n = 14$. CD56^{dim} subsets were analysed for linear trend with a repeated measures analysis of variance. ***$P < 0{\cdot}001$. (c) Mean subset distribution of all IFN-γ^{+} NK cells (upper pie chart) and all CD107a^{+} NK cells (lower pie chart) for all donors ($n = 14$) shown in (a) and (b).

Discussion

Vaccination typically provides long-lasting protection against infectious diseases by inducing the expansion and differentiation of small populations of naive, antigen-specific, T and B cells into much larger populations of long-lived memory cells with enhanced effector function. In particular, antigen-specific memory CD4^{+} T cells augment B-cell, CD8^{+} T-cell and macrophage-mediated effector functions.[22] Although circulating antibody may persist for many years after vaccination, frequencies of antigen-specific memory T cells are typically extremely low in peripheral blood (approximately 1 in 10 000[23]) and can be difficult to detect in the absence of recent boosting. However, the observation that IL-2 produced in an antigen-specific manner by CD4^{+} T cells can activate a substantial proportion (varying from ~ 1% up to 60% in some cases) of all circulating NK cells,[2,3,13,18,24] and that these responses can be detected for more than 20 years after vaccination in the case of DTP, suggests that NK cell responsiveness might represent a more sensitive biomarker of T-cell induction and maintenance and might therefore have a role to play in evaluation of new vaccines or new vaccine formulations. Whether NK cells – activated by T-cell IL-2 or by cross-linking of Fc receptors (CD16) by immune complexes – play an important role as effectors of vaccine-induced immunity is as yet unknown but the speed with which they are activated (within 6 hr of exposure to the pathogen[3]) and the large number of potentially responding cells suggest that their role should be investigated.

Here, we observed that NK cell responses to pertussis were significantly greater in magnitude than responses to DT or TT, even though all three antigens would have been administered together during vaccination. A likely explanation for this is that the pertussis antigen is a whole cell preparation containing numerous ligands for pattern recognition receptors on macrophages and dendritic cells, leading to their secretion of IL-12 and IL-18, which is necessary to induce NK cells to secrete IFN-γ

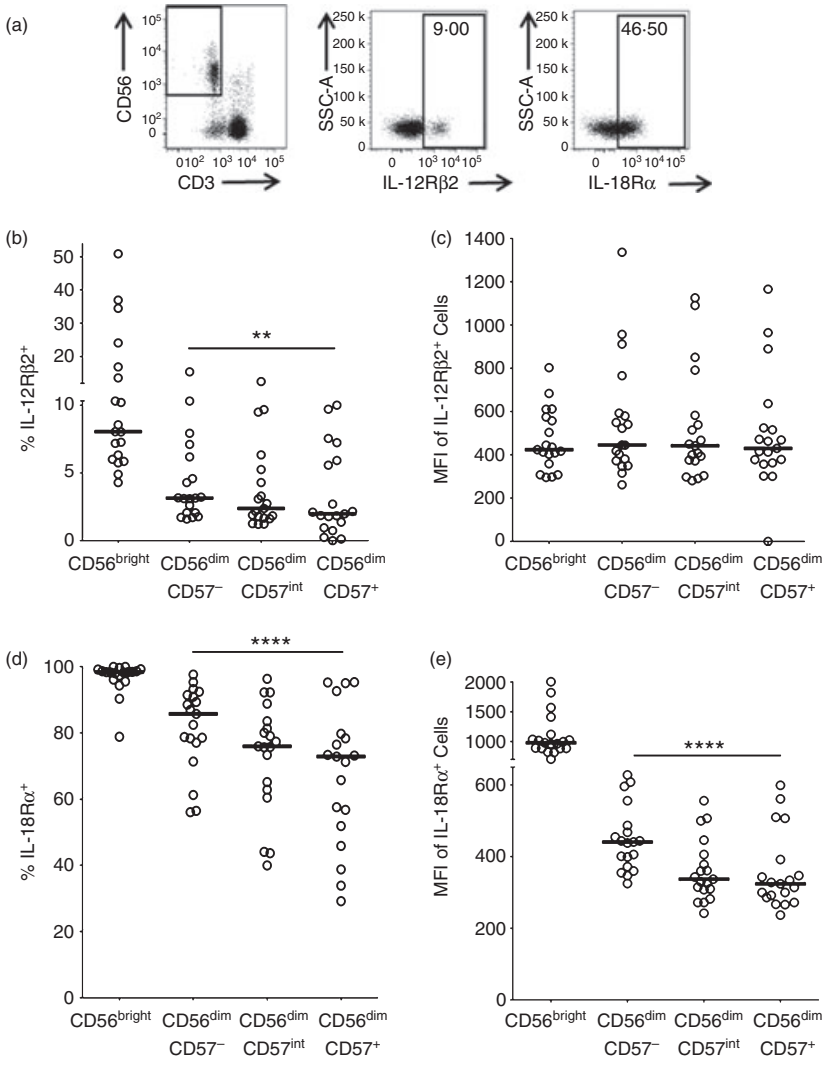

Figure 6. Interleukin-12 receptor $\beta 2$ (IL-12Rβ2) and IL-18Rα expression decrease with CD57 expression. Peripheral blood mononuclear cells were analysed *ex vivo* for IL-12Rβ2 and IL-18Rα expression. (a) Representative flow cytometry plots for IL-12Rβ2 and IL-18Rα. Frequency (b) and mean fluorescence intensity (MFI) (c) of IL-12Rβ2 expression, and frequency (d) and MFI (e) of IL-18Rα expression, were assessed by subset. Each data point represents one donor, $n = 19$. Lines indicate median values. $CD56^{dim}$ subsets were analysed for linear trend with a repeated measures analysis of variance. $^{****}P \leq 0{\cdot}0001$.

and become cytotoxic.[4,13] Purified toxoids such as DT and TT lack such ligands and so, *in vitro* at least, NK cells can only be induced to respond in the presence of exogenous IL-12 and IL-18. *In vivo*. however, infection by live tetanus and diphtheria bacteria would presumably induce a strong accessory cell cytokine response. On the other hand, much stronger NK responses to pertussis than DT or TT were seen even in the presence of LCC, suggesting that whole cell pertussis may also induce a stronger T-cell response than does a toxoid antigen.

Despite an overall tendency for NK cells to respond to vaccine antigens, there was considerable heterogeneity between individuals, which may in part be explained by inter-individual variation in T-cell IL-2 responses. However, we also observed heterogeneity between NK cell subsets in their responsiveness to vaccine-driven signals, with responses being dominated by $CD56^{bright}$ $CD57^{-}$ and $CD56^{dim}$ $CD57^{-}$ NK cells. This correlated with higher levels of CD25 expression on IL-12/IL-18-activated $CD57^{-}$ cells compared with $CD57^{+}$ cells and a higher resting level expression of IL-12Rβ2 and IL-18Rα on these cells. The relationship between NK cell phenotype and responsiveness to exogenous cytokines is summarized in Fig. 7. These findings are in line with previous reports that $CD57^{+}$ NK cells are less able to respond to cytokines,[10,12] and express lower levels of IL-18Rα and lower amounts of mRNA for IL-12Rβ2, compared with $CD57^{-}$ NK cells. IL-18 is known to induce expression of the high-affinity IL-2Rα (CD25) on NK cells[25] whereas IL-12 is necessary, but not sufficient, for their production of

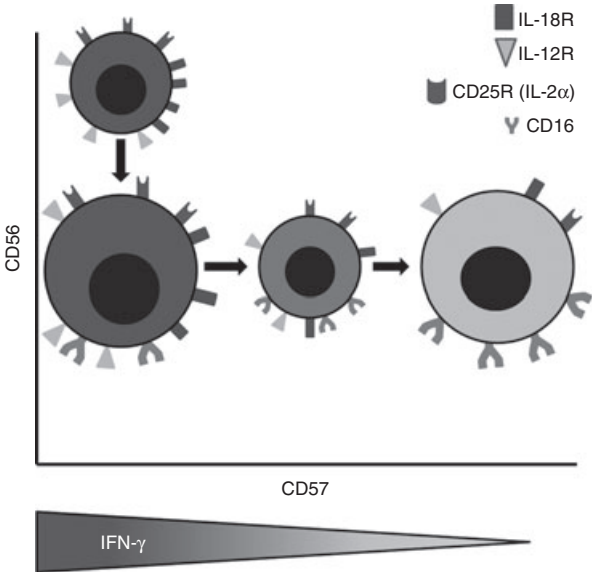

Figure 7. The relationship between natural killer (NK) cell phenotype and functional responses to exogenous cytokines. NK cell subsets defined by CD56 and CD57 expression also differ in their expression of interleukin-18 receptor α (IL-18Rα), IL-12Rβ2 and CD16 and in their ability to up-regulate CD25. Declining expression of cytokine receptors with increasing expression of CD57 results in gradual loss of the ability of the cells to secrete interferon-γ (IFN-γ) after cytokine stimulation. The diameter of each cell reflects the approximate proportion of the entire NK population belonging to that subset. The shading of each cell reflects the capacity of that subset to produce IFN-γ in response to high-dose IL-12 + IL-18 (darkest shading denotes the highest IFN-γ production). NB: Our data indicate that CD107a expression does not differ significantly between subsets, whether induced by exogenous cytokines or receptor cross-linking.

IFN-γ.[26] Moreover, IL-2 induces expression of the inducible chain of the IL-12R (IL-12β2).[27] Thus, as shown here, synergy between these three cytokine signals, IL-2, IL-12 and IL-18, results in NK cells producing high levels of IFN-γ during the first 18–24 hr following re-exposure to vaccine antigens.

Interestingly, we have observed that the maturation of NK cells from CD56^{bright} CD57$^-$ to CD56^{dim} CD57$^+$ is a gradual process with functional changes being highly correlated with CD56 and CD57 expression. This is particularly apparent for the cytokine-driven pathway of NK cell activation where expression of IL-12R and IL-18R as well as IL-12/IL-18-induced CD25 expression and IFN-γ synthesis are all very tightly negatively associated with CD57 expression. We find that CD57^{int} NK cells make significant amounts of IFN-γ after stimulation with high-dose IL-12/IL-18 but respond less robustly to low concentration cytokines and vaccine antigens, suggesting that they may fail to compete effectively with CD57$^-$ NK cells when cytokines are limiting.

An area of increasing concern in industrialized countries is the burden of infectious disease and poor response to vaccination in the elderly population.[28] Although ageing in the innate immune system, including age-associated changes in the composition, phenotype and function of circulating NK cells, is being linked to increased susceptibility to *de novo* viral and bacterial infections,[29] deterioration of antigen-specific memory responses and reduced responsiveness to vaccination with increasing age tend to be attributed to narrowing of the T-cell repertoire and functional senescence of the T-cell pool.[30,31] Our data suggest, however, that these two components of immune ageing may interact; deteriorating CD4$^+$ T-cell responses will limit the availability of IL-2 to drive NK cell responses while, at the same time, the proportion of CD57$^-$ NK cells able to respond to IL-2 will decrease. We predict, therefore, that vaccination-induced NK cell IFN-γ responses could decline with increasing age, potentially contributing to reduced vaccine efficacy in elderly populations. In addition, subclinical human cytomegalovirus (HCMV) infections may potentiate the functional differentiation and senescence of NK cells.[9,32–35] Given that at least 40% of the world population is HCMV seropositive, and prevalence can exceed 95% in some African and Asian populations,[36] HCMV exposure may contribute significantly to poor vaccine efficacy at a population level. Studies to test these various predictions are currently underway in our laboratory.

Acknowledgements

We thank Carolynne Stanley for subject recruitment and blood sample collection and Elizabeth King for assistance with flow cytometry. This study was funded by the UK Medical Research Council (G1000808) and Carolyn Nielsen is supported by an MRC PhD Studentship in Vaccine Research (MR/J003999/1).

Disclosures

The authors declare that they have no competing financial interests.

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LONDON SCHOOL OF HYGIENE
& TROPICAL MEDICINE

ETHICS COMMITTEE

APPROVAL FORM
Application number: **5520**

Name of Principal Investigator	**Eleanor Riley**
Department	**Infectious and Tropical Diseases**
Head of Department	**Professor Simon Croft**

Title: **To set up a system of voluntary blood donation at the London School of Hygiene & Tropical Medicine**

This application is approved by the Committee.

Chair of the Ethics Committee ..... T. W. Meade .....

Date .....21 April 2009.....

Approval is dependent on local ethical approval having been received.

Any subsequent changes to the application must be submitted to the Committee via an E2 amendment form.

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Observational / Interventions Research Ethics Committee

Eleanor Riley
Professor of Immunology
IID/ITD
LSHTM

7 September 2012

Dear Professor Riley,

Study Title: **The role of Natural Killer cells in Immunity induced by influenza vaccines**
LSHTM ethics ref: **6237**

Thank you for your letter of 15 August 2012, responding to the Interventions Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered by the Committee.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

Approval is dependent on local ethical approval having been received, where relevant.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	Version	Date
LSHTM ethics application	n/a	10/07/2012
Protocol	V3	03/07/2012
Information Sheet	V4	14/08/2012
Consent form	V2	17/05/2012
Sample recruitment email	V2	14/08/2012

After ethical review

Any subsequent changes to the application must be submitted to the Committee via an E2 amendment form. All studies are also required to notify the ethics committee of any serious adverse events which occur during the project via form E4. An annual report form (form E3) is required on the anniversary of the approval of the study and should be submitted during the lifetime of the study. At the end of the study, please notify the committee via form E5.

Yours sincerely,

<Not signed to avoid delay>

Dr Wenzel Geissler
Anthropologist
Member of Committee and Acting Chair for Application
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http://intra.lshtm.ac.uk/management/committees/ethics/

Non WHO Reference Material
Tetanus Toxoid (Non-Adsorbed)
NIBSC code: 02/232
Instructions for use
(Version 9.0, Dated 20/01/2014)

This material is not for in vitro diagnostic use.

1. INTENDED USE

Tetanus toxoid was provided to NIBSC by Aventis Pasteur MSD, France. The product was freeze-dried in a medium containing glycine in November 2002. It is been confirmed as suitable for use as a control antigen in immunodiffusion identity assays.

2. CAUTION

This preparation is not for administration to humans.

The material is not of human or bovine origin. As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

3. UNITAGE

Each ampoule contains 900 Lf units of tetanus toxoid, non-adsorbed.

4. CONTENTS

Country of origin of biological material: France.
The material is purified tetanus toxoid, of purity >1000 Lf/mg pN, stabilized with glycine. The material was provided by Aventis Pasteur MSD in one glass bottle containing 800 ml of toxoid with an internal code number FA082448, with specifications of 5000 Lf/ml (4.42 mg protein Nitrogen (25 mg/ml protein by BCA assay). The product fully meets PhEur specifications for purity, safety and toxicity/toxicity reversal for use in manufacturing of adsorbed vaccines. Material (750 ml) was diluted 1/5 with 400 ml of 1M sodium chloride (0.1 M final concentration), 2000 ml of 10% glycine (5% final concentration) and 850 ml distilled water, and 1.0 ml was filled into ampoules for freeze-drying. The average weight of the ampoule content was determined as 0.0631 g of dry weight ± 0.38%. The residual moisture is less than 1% and samples measured were in the range 0.07% to 0.6%.

5. STORAGE

Unopened ampoules should be stored at -20°C.
Please note: because of the inherent stability of lyophilized material, NIBSC may ship these materials at ambient temperature.

6. DIRECTIONS FOR OPENING

DIN ampoules have an 'easy-open' coloured stress point, where the narrow ampoule stem joins the wider ampoule body.
Tap the ampoule gently to collect the material at the bottom (labeled) end. Ensure that the disposable ampoule safety breaker provided is pushed down on the stem of the ampoule and against the shoulder of the ampoule body. Hold the body of the ampoule in one hand and the disposable ampoule breaker covering the ampoule stem between the thumb and first finger of the other hand. Apply a bending force to open the ampoule at the coloured stress point, primarily using the hand holding the plastic collar.
Care should be taken to avoid cuts and projectile glass fragments that might enter the eyes, for example, by the use of suitable gloves and an eye shield. Take care that no material is lost from the ampoule and no glass falls into the ampoule. Within the ampoule is dry nitrogen gas at slightly less than atmospheric pressure. A new disposable ampoule breaker is provided with each DIN ampoule.

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution
The entire contents of each ampoule should be completely resuspended in an accurately measured amount of a suitable solution (e.g. saline). A suspension of the total content of an ampoule will contain 900 Lf in the total volume. The suspension should kept at 4°C and should not be frozen.

8. STABILITY

Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference Materials should be stored on receipt as indicated on the label.

When stored unopened at the recommended temperature (-20°C), the freeze-dried material is highly stable with a predicted degradation rate of 0.003% loss of activity per year [2].

Once reconstituted, 02/232 has been confirmed to be stable for up to 12 months in in vitro assays at NIBSC following storage at +4°C. However, users are encouraged to determine the stability of the material according to their own methods of preparation, storage and use.

Users who have data supporting any changes in the characteristics of this material are encouraged to contact NIBSC.

9. REFERENCES

1. Preneta-Blanc, R., Rigsby, P., Sloth Wilhelmsen, E., Tierney, R., Brierley, M. and Sesardic, D. 2007. Collaborative Study: Calibration of Replacement International Standard of Tetanus Toxoid for use in Flocculation Test. WHO Expert Committee on Biological Standardization. WHO/BS/07.2061.
2. Preneta-Blanc, R., Rigsby, P., Sloth-Wilhelmsen, E., Tierney, R., Brierley, M. and Sesardic, D. Calibration of replacement international standards of diphtheria and tetanus for use in flocculation test. Biologicals, 36 (2008), 315-326.

10. ACKNOWLEDGEMENTS

N/A

11. FURTHER INFORMATION

Further information can be obtained as follows;
This material: enquiries@nibsc.org
WHO Biological Standards:
http://www.who.int/biologicals/en/
JCTLM Higher order reference materials:
http://www.bipm.org/en/committees/jc/jctlm/
Derivation of International Units:
http://www.nibsc.org/products/biological_reference_materials/frequently_asked_questions/how_are_international_units.aspx
Ordering standards from NIBSC:
http://www.nibsc.org/products/ordering_information/frequently_asked_questions.aspx
NIBSC Terms & Conditions:
http://www.nibsc.org/terms_and_conditions.aspx

12. CUSTOMER FEEDBACK

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13. CITATION

In all publications, including data sheets, in which this material is referenced, it is important that the preparation's title, its status, the NIBSC code number, and the name and address of NIBSC are cited and cited correctly.

14. MATERIAL SAFETY SHEET

Physical and Chemical properties	
Physical appearance: Freeze-dried powder	Corrosive: No
Stable: Yes	Oxidising: No
Hygroscopic: No	Irritant: No
Flammable: No	Handling: See caution, Section 2
Other (specify): Chemically inactivated tetanus toxin. Tested and found to be free of active toxin and free from ability to reverse to toxin.	
Toxicological properties	
Effects of inhalation: Not established, avoid inhalation	
Effects of ingestion: Not established, avoid ingestion	
Effects of skin absorption: Not established, avoid contact with skin	
Suggested First Aid	
Inhalation: Seek medical advice	
Ingestion: Seek medical advice	
Contact with eyes: Wash with copious amounts of water. Seek medical advice	
Contact with skin: Wash thoroughly with water.	
Action on Spillage and Method of Disposal	
Spillage of ampoule contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.	

15. LIABILITY AND LOSS

In the event that this document is translated into another language, the English language version shall prevail in the event of any inconsistencies between the documents.

Unless expressly stated otherwise by NIBSC, NIBSC's Standard Terms and Conditions for the Supply of Materials (available at http://www.nibsc.org/About_Us/Terms_and_Conditions.aspx or upon request by the Recipient) ("Conditions") apply to the exclusion of all other terms and are hereby incorporated into this document by reference. The Recipient's attention is drawn in particular to the provisions of clause 11 of the Conditions.

16. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 1.0 ml
Toxicity Statement: Non-toxic
Veterinary certificate or other statement if applicable**.** **Attached:** No

Non WHO Reference Material
Diphtheria Toxoid, Non-adsorbed
NIBSC code: 69/017
Instructions for use
(Version 8.0, Dated 29/11/2012)

This material is not for in vitro diagnostic use.

1. INTENDED USE

This material has been prepared by NIBSC in 1969 as a freeze dried preparation containing diphtheria toxoid without adjuvant (non-adsorbed). The toxoid was provided by Glaxo Laboratories in May 1968 with the intention of making a European reference standard. However this standard was never established and this material has not been calibrated in a collaborative study. This material is not calibrated in International Units and is therefore not suitable for use as a standard for determining the potency of non-adsorbed diphtheria vaccines.

2. CAUTION

This preparation is not for administration to humans.

The material is not of human or bovine origin. As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

3. UNITAGE

The Lf content was determined at NIBSC by quantitative Immunodiffusion assay against the International Standard of Diphtheria Toxoid for use in Flocculation Test (02/176). The diphtheria toxoid content of each ampoule was determined as 445 Lf. Total protein content was determined as 1 mg/ampoule by BCA protein assay.

4. CONTENTS

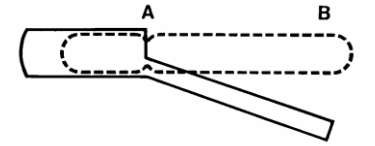
Country of origin of biological material: United Kingdom.
The purified diphtheria toxoid contained 1500 Lf/mg pN on arrival. Glycine was added at 22.5 g/L before freeze drying 1.0 ml of liquid per ampoule. The average weight of the ampoule content was determined as 1.014 g of dry wt. +/- 0.7%.

5. STORAGE

Unopened ampoules should be stored at -20°C
Please note: because of the inherent stability of lyophilized material, NIBSC may ship these materials at ambient temperature.

6. DIRECTIONS FOR OPENING

Tap the ampoule gently to collect the material at the bottom (labelled) end. Ensure ampoule is scored all round at the narrow part of the neck, with a diamond or tungsten carbide tipped glass knife file or other suitable implement before attempting to open. Place the ampoule in the ampoule opener, positioning the score at position 'A'; shown in the diagram below. Surround the ampoule with cloth or layers of tissue paper. Grip the ampoule and holder in the hand and squeeze at point 'B'. The ampoule will snap open. Take care to avoid cuts and projectile glass fragments that enter eyes. Take care that no material is lost from the ampoule and that no glass falls into the ampoule.



Side view of ampoule opening device containing an ampoule positioned ready to open. 'A' is the score mark and 'B' the point of applied pressure.

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution

The entire contents of each ampoule should be completely dissolved with 1 ml of distilled water prior to use. The resulting concentrate may be diluted further as required. The ampoule contents contain no bacteriostat but have been tested and found sterile after freeze drying.

This preparation can be used in assays designed to detect diphtheria toxoid in vaccines using immunodetection methods. It is the responsibility of the recipient to establish the usefulness of this preparation for the purposes they wish to use it for. NIBSC takes no responsibility for the use of this preparation for any purpose as the preparation has not been calibrated in a collaborative study and has no official status.

8. STABILITY

Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference Materials should be stored on receipt as indicated on the label.

There is no data available on long term stability. However dried toxoid standards are expected to undergo negligible loss of potency during long term storage at the indicated storage temperature [1].

Once reconstituted, users should determine the stability of the material according to their own method of preparation, storage and use. Users who have data supporting any deterioration in the characteristics of any reference preparation are encouraged to contact NIBSC.

9. REFERENCES

1. Lyng J. Quantitative Estimation of Diphtheria and Tetanus Toxoids. 4. Toxoids as International Reference Materials Defining Lf-units for Diphtheria and Tetanus Toxoids. Biologicals, 1990 vol. 18, pp 11-17.

10. ACKNOWLEDGEMENTS

N/A

11. FURTHER INFORMATION

Further information can be obtained as follows;
This material: enquiries@nibsc.org
WHO Biological Standards:
http://www.who.int/biologicals/en/
JCTLM Higher order reference materials:
http://www.bipm.org/en/committees/jc/jctlm/
Derivation of International Units:
http://www.nibsc.org/products/biological_reference_materials/frequently_asked_questions/how_are_international_units.aspx
Ordering standards from NIBSC:
http://www.nibsc.org/products/ordering_information/frequently_asked_questions.aspx
NIBSC Terms & Conditions:
http://www.nibsc.org/terms_and_conditions.aspx

12. CUSTOMER FEEDBACK

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13. CITATION

In all publications, including data sheets, in which this material is referenced, it is important that the preparation's title, its status, the NIBSC code number, and the name and address of NIBSC are cited and cited correctly.

14. MATERIAL SAFETY SHEET

Physical and Chemical properties	
Physical appearance: Freeze-dried powder	Corrosive: No
Stable: Yes	Oxidising: No
Hygroscopic: Yes	Irritant: No
Flammable: No	Handling: See caution, Section 2
Other (specify):	Contains material of bacterial origin
Toxicological properties	
Effects of inhalation:	Not established, avoid inhalation
Effects of ingestion:	Not established, avoid ingestion
Effects of skin absorption:	Not established, avoid contact with skin
Suggested First Aid	
Inhalation:	Seek medical advice
Ingestion:	Seek medical advice
Contact with eyes:	Wash with copious amounts of water. Seek medical advice
Contact with skin:	Wash thoroughly with water.
Action on Spillage and Method of Disposal	
Spillage of ampoule contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.	

15. LIABILITY AND LOSS

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16. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 1 g
Toxicity Statement: Non-toxic
Veterinary certificate or other statement if applicable**.** **Attached:** No

Non WHO Reference Material
Bordetella pertussis (Whole cell vaccine) 3 BRP
NIBSC code: 88/522
Instructions for use
(Version 6.0, Dated 09/04/2013)

This material is not for in vitro diagnostic use.

1. INTENDED USE

This *Bordetella pertussis* preparation, coded 88/522, has been established as the third British Reference Preparation for Pertussis (whole cell) Vaccine potency.

2. CAUTION

This preparation is not for administration to humans.

The material is not of human or bovine origin.

3. UNITAGE

50 International Units per ampoule.

4. CONTENTS

Country of origin of biological material:United Kingdom.
Each ampoule contains the freeze dried residue of 1.0 ml of an aqueous solution which contained :-

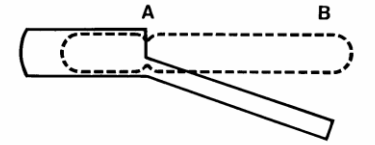
Dextran (90kD)	80mg
Glucose	50mg
B. pertussis	20 x 10^{10} organisms

5. STORAGE

Unopened ampoules should be stored at -20°C.
Please note: because of the inherent stability of lyophilized material, NIBSC may ship these materials at ambient temperature.

6. DIRECTIONS FOR OPENING

Tap the ampoule gently to collect the material at the bottom (labelled) end. Ensure ampoule is scored all round at the narrow part of the neck, with a diamond or tungsten carbide tipped glass knife file or other suitable implement before attempting to open. Place the ampoule in the ampoule opener, positioning the score at position 'A'; shown in the diagram below. Surround the ampoule with cloth or layers of tissue paper. Grip the ampoule and holder in the hand and squeeze at point 'B'. The ampoule will snap open. Take care to avoid cuts and projectile glass fragments that enter eyes. Take care that no material is lost from the ampoule and that no glass falls into the ampoule.



Side view of ampoule opening device containing an ampoule positioned ready to open. 'A' is the score mark and 'B' the point of applied pressure.

7.USE OF MATERIAL

The *B. pertussis* suspension was generously donated by Wellcome Biotech, Beckenham, UK through the good offices of Mr P. Knight.
The bacteria were grown and killed using standard methods and contained agglutinogens1 ,2 and 3.
Ampoules coded 88/522 were prepared according to the procedures used for International Standards (29th ECBS Report 1978). The bacteria were suspended at 20 x 10^{10} cells/ml in a solution of 8% dextran (90kD) and 5% glucose. The suspension was distributed in 1.0ml aliquots into ampoules. The ampouled suspension was lyophilised and the ampoules sealed under nitrogen by heat fusion of the glass and stored at -20°C in the dark.

Collaborative Study
Nine laboratories in seven countries participated in a collaborative study to evaluate 88/522 as a reference preparation for pertussis vaccine potency. The study showed that :-
1.The intra and inter-laboratory variability with respect to the potency assay was in agreement with that shown in previous studies.
2.Similar estimates of potency were obtained for 88/522 interms of both the 2nd British Reference Preparation and the 2nd International Standard.
3 That 88/522 was suitable for establishment and that it be assigned the potency of 50 IU / ampoule.

8. STABILITY

Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference Materials should be stored on receipt as indicated on the label.

Users who have data supporting any deterioration in the characteristics of any reference preparation are encouraged to contact NIBSC.

9. REFERENCES

Redhead K. Das RG
A collaborative assay on the proposed 3rd British Reference Preparation for pertussis vaccine and the relative potencies of the 2nd IS and the 2nd British Reference Preparation for pertussis vaccine.
Biologicals 1991, 19 : 107 - 111

10. ACKNOWLEDGEMENTS

Grateful acknowledgements are due to Mr P Knight and Wellcome Biotech for providing the material; the participants in the collaborative study; and Standards Processing Division for the filling.

11. FURTHER INFORMATION

Further information can be obtained as follows;
This material: enquiries@nibsc.org
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Derivation of International Units:
http://www.nibsc.org/products/biological_reference_materials/frequently_asked_questions/how_are_international_units.aspx
Ordering standards from NIBSC:
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14. MATERIAL SAFETY SHEET

Physical and Chemical properties	
Physical appearance:Freeze dried powder	Corrosive: No
Stable: Yes	Oxidising: No
Hygroscopic: No	Irritant: No
Flammable: No	Handling: See caution, Section 2
Other (specify):	Contains material of biological origin
Toxicological properties	
Effects of inhalation:	Not established, avoid inhalation
Effects of ingestion:	Not established, avoid ingestion
Effects of skin absorption:	Not established, avoid contact with skin
Suggested First Aid	
Inhalation:	Seek medical advice
Ingestion:	Seek medical advice
Contact with eyes:	Wash with copious amounts of water. Seek medical advice
Contact with skin:	Wash thoroughly with water.
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Spillage of ampoule contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water. Absorbent materials used to treat spillage should be treated as biological waste.	

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Country of origin for customs purposes*:United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 1.0 - 2.0 g
Toxicity Statement: Non-toxic
Veterinary certificate or other statement if applicable**.** **Attached:**No

Regular Article

IMMUNOBIOLOGY

Rapid NK cell differentiation in a population with near-universal human cytomegalovirus infection is attenuated by NKG2C deletions

Martin R. Goodier,[1] Matthew J. White,[1] Alansana Darboe,[1,2] Carolyn M. Nielsen,[1] Adriana Goncalves,[3] Christian Bottomley,[4] Sophie E. Moore,[2,5] and Eleanor M. Riley[1]

[1]Department of Immunology and Infection, London School of Hygiene and Tropical Medicine, London, United Kingdom; [2]Medical Research Council International Nutrition Group, Medical Research Council Keneba, Medical Research Council Unit, The Gambia; [3]Department of Clinical Research and [4]Department of Infectious Disease Epidemiology, London School of Hygiene and Tropical Medicine, London, United Kingdom; and [5]Medical Research Council Human Nutrition Research, Cambridge, United Kingdom

Key Points

- HCMV infection in early life is associated with rapid phenotypic and functional differentiation of NK cells.
- Emergence of CD57$^+$ NK cells is attenuated in children lacking *NKG2C*.

Natural killer (NK) cells differentiate and mature during the human life course; human cytomegalovirus (HCMV) infection is a known driver of this process. We have explored human NK cell phenotypic and functional maturation in a rural African (Gambian) population with a high prevalence of HCMV. The effect of age on the frequency, absolute number, phenotype, and functional capacity of NK cells was monitored in 191 individuals aged from 1 to 49 years. Increasing frequencies of NK cells with age were associated with increased proportions of CD56^{dim} cells expressing the differentiation marker CD57 and expansion of the NKG2C$^+$ subset. Frequencies of NK cells responding to exogenous cytokines declined with age in line with a decreased proportion of CD57$^-$ cells. These changes coincided with a highly significant drop in anti-HCMV IgG titers by the age of 10 years, suggesting that HCMV infection is brought under control as NK cells differentiate (or vice versa). Deletion at the *NKG2C* locus was associated with a gene dose-dependent reduction in proportions of CD94$^+$ and CD57$^+$ NK cells. Importantly, anti-HCMV IgG titers were significantly elevated in *NKG2C*$^{-/-}$ children, suggesting that lack of expression of NKG2C may be associated with altered control of HCMV in childhood. (*Blood*. 2014;124(14):2213-2222)

Introduction

Natural killer (NK) cells play essential roles in controlling infection and surveillance for damaged, dysfunctional, or neoplastic cells.[1] NK cells differentiate during the human life course. CD56^{bright} cells are the least-differentiated population of peripheral blood NK cells, expressing c-kit and high levels of the c-type lectin-like receptor CD94/NKG2A, CD62L, and natural cytotoxicity receptors (NCRs) NKp30 and NKp46, and lacking expression of killer cell immunoglobulin-like receptors (KIR), CD16, and CD57.[2-5] CD56^{bright} NK cells express cytokine receptors and produce interferon (IFN)-γ in response to cytokines. In contrast, CD56^{dim} cells express FcγRIII(CD16); express varying levels of CD94/NKG2A, KIR, NCRs, and perforin; retain their ability to secrete IFN-γ; and have higher cytotoxic capacity.[3] Heterogeneity within the CD56^{dim} subset is associated with acquisition of CD57.[2,4,5] CD56^{dim}CD57$^-$ NK cells are phenotypically and functionally similar to CD56^{bright} cells, whereas CD56^{dim}CD57$^+$ cells produce little IFN-γ and have shorter telomeres and lower proliferative capacity,[5,6] but degranulate extensively after crosslinking of CD16.[2,5] Acquisition of CD57 is associated with onset of expression of NKG2C, although the codependence of these events and their implications for function are not understood.[7,8]

Although the external drivers of NK cell differentiation are incompletely understood, inflammation, associated with infection or loss of immune homeostasis, plays a key role.[9] This view is supported by evidence that the late differentiation marker, CD57, can be induced on NK cells by high concentrations of IL-2,[5] that NKG2C$^+$ NK cells can be expanded by coculture with human cytomegalovirus (HCMV)-infected fibroblasts,[10] that HCMV-seropositive individuals have increased frequencies of NKG2C$^+$ NK cells,[10-13] and that there is rapid expansion of CD57$^+$NKG2C^{hi} NK cells during acute HCMV infection[14] and in individuals infected with Epstein Barr virus (EBV),[7] hantavirus,[15] hepatitis viruses,[16] and chikungunya virus.[17]

Among Caucasians, NK cell maturation is highly age-dependent. Marked phenotypic and functional differences are observed between NK populations in cord blood, in young children, in adults, and in elderly individuals.[18-22] Young children have higher frequencies of CD56^{bright}CD16$^-$ and NKG2A$^+$NKG2C$^-$ NK cells compared with adults, and younger adults have higher frequencies of these cells compared with the elderly.[18-22] Moreover, NCR$^+$ and NKG2D$^+$ NK cells decrease in frequency with increasing age, concomitant with loss of CD62L and acquisition of CD57.[2,4,18,22] NK cell cytokine production decreases with increasing age, but cytotoxic responses are conserved.[9,20,23] There is, however, a lack of data from older children and teenagers.

Submitted May 23, 2014; accepted August 12, 2014. Prepublished online as *Blood* First Edition paper, August 22, 2014; DOI 10.1182/blood-2014-05-576124.

The online version of this article contains a data supplement.

There is an Inside *Blood* Commentary on this article in this issue.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

Table 1. Cohort characteristics

Age group, years	n (male/female)	HCMV IgG$^+$, n (%)	HCMV IgG titer, IU/mL, median (range)	EBV nuclear antigen IgG$^+$, n (%)	EBV nuclear antigen IgG titer, IU/mL, median (range)	*NKG2C* genotype, n (%)*		
						+/+	+/−	−/−
1-2	23 (9/14)	20 (86.9)	487.5 (81.8-845.2)†	12 (52.2)	107.0 (48.5-178.6)	11 (47.8)	10 (43.4)	2 (8.7)
3-5	19 (6/13)	18 (94.7)	288.4 (80.9-1681.8)	13 (68.4)	134.0‡ (32.5-328.7)	7 (37.8)	10 (52.6)	2 (10.5)
6-9	18 (11/7)	18 (100)	361.1 (89.2-2200.2)¶	16 (88.9)	103.6 (33.1-219.7)	8 (47.0)	7 (41.2)	2 (11.8)
10-12	20 (10/10)	20 (100)	215.4 (43.4-1693.6)	18 (90.0)	119.3§ (37.2-359.5)	8 (44.4)	8 (44.4)	2 (11.1)
13-15	23 (10/13)	23 (100)	252.6 (51.5 −1057.9)	16 (70.0)	114.6 (29.7-193.4)	11 (47.8)	10 (43.4)	2 (8.7)
16-19	23 (11/12)	23 (100)	177.6 (61.2-678.1)	18 (78.2)	99.9 (23.9-195.2)	10 (47.6)	8 (38.1)	3 (14.3)
20-25	22 (11/11)	22 (100)	252.5 (81.5-828.4)	19 (86.4)	93.9 (27.6-171.7)	11 (52.4)	8 (38.1)	2 (9.5)
26-39	22 (13/9)	22 (100)	165.9 (39.0-968.4)	19 (86.4)	88.8 (24.9-272.7)	14 (73.7)	3 (15.7)	2 (10.5)
40-49	21 (10/11)	21 (100)	191.2 (53.5-735.2)	13 (61.9)	73.4 (24.0-183.2)	14 (70.0)	4 (20.0)	2 (10.0)
Total	191 (91/100)	187 (97.9)	252.6 (39-2200.1)	145 (75.9)	101.8 (23.9-359.5)	94 (51.9)	68 (37.6)	19 (10.5)

*NKG2C genotypes were obtained from a total of 181 individuals.
†Significantly higher anti-HCMV IgG titers compared with 16- to 19-year-olds and all groups older than 26 years; $P < .05$, analysis of variance.
‡Significantly elevated anti-EBV nuclear antigen IgG titers compared with all groups older than 16 years.
¶Significantly higher anti-HCMV IgG titers compared with all groups older than 16 years; $P < 0.01$, analysis of variance.
§Significantly elevated anti-EBV nuclear antigen IgG titers compared with all groups older than 20 years.

The extent to which NK cell differentiation is explained by either aging, per se, or by cumulative exposure to infection is unclear. Among allogeneic hematopoietic stem cell transplant recipients, the first wave of repopulating NK cells comprises predominantly CD56^{bright} or CD56^{dim}CD94$^+$cells; KIR$^+$ and CD57$^+$ cells can take up to 1 year to emerge.[2,24] However, among patients who reactivate HCMV after transplantation, NKG2C$^+$CD57$^+$ NK cells can be detected within 3 months, and the host's pretransplantation repertoire is fully reconstituted within 6 months, suggesting that exposure to infection is a significant determinant of NK cell maturation rates.[24-26]

Together, these data suggest that age-related changes in NK cell phenotype and function may be modified by the infection status of the host and that rates of change across populations may depend on the prevalence of particular infections. If so, the prevalence of infections such as HCMV may have far-reaching implications for risk for other infections, cancers, or autoimmune disease. To begin to address this important aspect of NK cell biology, we have characterized NK cell phenotype and function in an African population that is itself characterized by a high burden of infectious disease, including near-universal HCMV infection.

Materials and methods

Study subjects

This study was approved by the ethical review committees of the Gambia Government/Medical Research Council and the London School of Hygiene and Tropical Medicine. Participants were recruited from the villages of Keneba, Manduar, and Kantong Kunda in the West Kiang district, The Gambia. After fully informed consent was obtained in accordance with the Declaration of Helsinki, including parental/guardian consent for minors, venous blood samples were collected from 191 individuals aged 1 to 49 years. Individuals with signs or symptoms of current disease or who were known to be pregnant or infected with HIV were excluded. Plasma was screened for IgG against HCMV(BioKit), tetanus toxoid (Holzel Diagnostica), hepatitis B surface antigen (Diasorin), and EBV nuclear antigen (Euroimmun). Subject characteristics are shown in Table 1.

Peripheral blood mononuclear cell preparation and culture

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation (Histopaque, Sigma) and analyzed ex vivo and after 18-hour culture with cytokines (5 ng/mL rhIL-12; Peprotec) plus 50 ng/mL rhIL-18 (R&D Systems) or with K562 cells (an Effector:Target ratio of 2:1). Fluorescein isothiocyanate (FITC)-conjugated anti-CD107a (BD Biosciences) was added throughout the culture. Brefeldin A and Monensin (BD Biosciences) were added after 15 hours.

Flow cytometry

PBMCs were incubated with combinations of the following monoclonal antibodies: anti-CD3-V500, anti-CD56-PeCy7 and anti-CD94-FITC, anti-NKG2C-PE and anti-NKG2A-APC, anti-CD8-PeCy7, anti-CD57-e450 and anti-CD16-APC-e780 or APC, anti-CD4-PE and anti-CD45RA-APC-H7, anti-CD8-PeCy7, anti-CD27-FITC, anti-CD28-PeCy7 and anti-CCR7-APC, anti-CD45-FITC, anti-CD11c-PE, anti-CD19-PeCy5, anti-CD123-efluor450 and anti-CD14-APCe780, anti-CD107a-FITC, anti-CD25PE, and anti-IFN-γ-APC-efluor780 (supplemental Methods, available on the *Blood* Web site). Cells were acquired on a LSRII® flow cytometer using FacsDiva® software. Data analysis was performed using FlowJo® (TreeStar).

NKG2C genotyping

DNA was extracted from whole blood (Wizard genomic DNA extraction kit, Promega), and the *NKG2C* genotype was determined by touchdown PCR (Phusion® High Fidelity PCR kits, New England Biolabs).[27] PCR primers and conditions are described in the supplemental Methods.

Statistical analysis

Statistical analysis was performed using Statview and Stata version 13.1. Nonlinear effects of age were modeled using natural cubic splines in linear regression models; P values (F-test) and R^2 values were obtained from these models. One-way analysis of variance was used to compare responses of individuals of different genotypes. Differences between NK cell subsets were compared using Wilcoxon signed rank tests.

Results

High rates of HCMV and EBV infection in the study population

HCMV infection rates are high in Africa, and thus, as expected, only 4 of the 191 individuals were HCMV-seronegative; seronegative patients were aged between 1 and 3 years, suggesting universal HCMV infection within the first 3 years of life (Table 1). Interestingly, anti-HCMV antibody titers were significantly higher among those younger than 10 years than in older individuals, suggesting that optimal control of HCMV infection takes some years to develop (Table 1). EBV infection was also common, with 75% of the cohort being seropositive for EBV nuclear antigen. EBV nuclear

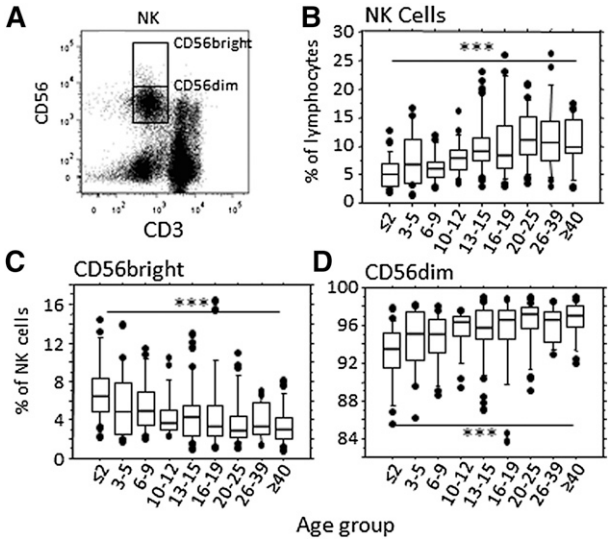

Figure 1. Age-related changes in NK cell frequencies (A). NK cells were identified within PBMC after gating on singlets and viable lymphocytes. $CD56^{+}CD3^{-}$ NK cells were then subsequently gated into $CD56^{bright}$ and $CD56^{dim}$ subsets. Frequencies of (B) all NK cells, (C) $CD56^{bright}$, and (D) $CD56^{dim}$ NK cells are shown for each age group. Horizontal bars represent median values, boxes extend from the 25th to the 75th percentile, and whiskers represent the 95th percentiles. Asterisks represent significant trends across the entire cohort (*$P < .05$, ***$P < .001$, *F*-test).

antigen seropositivity rates were lowest in children 2 years old or younger, and anti-EBV nuclear antigen titers tended to be higher in those younger than 15 years than in older individuals (Table 1).

NK cell numbers and frequencies change with age

NK cell numbers and frequencies and the distribution of $CD56^{bright}$ and $CD56^{dim}$ subsets (Figure 1A) were analyzed by age group (Figure 1). Consistent with previous observations,[18-22] the proportion of NK cells among peripheral blood lymphocytes increased significantly with age, reaching a plateau at approximately 15 years (Figure 1B). Within the total NK cell population, the proportion of $CD56^{bright}$ NK cells declined significantly with increasing age (Figure 1C), and the proportion of $CD56^{dim}$ cells increased (Figure 1D), with subset distribution stabilizing at approximately 10 to 12 years (supplemental Figure 1). The absolute number of peripheral blood $CD56^{bright}$ and $CD56^{dim}$ NK cells declined with age, indicating that the increased frequency of $CD56^{dim}$ cells in older individuals was not sufficient to offset the overall decline in NK cell numbers (supplemental Figure 1; supplemental Table 1).

These early and very marked changes in NK cell phenotype contrasted with more gradual changes in T-cell phenotype (supplemental Figure 2). Consistent with previous studies,[28,29] we observed a steady decline in naive $CD4^{+}$ and $CD8^{+}$ T-cell frequencies, with a parallel increase in frequencies of effector memory and central memory T cells. However, in contrast to published data,[28,29] the frequency of terminally differentiated effector memory (TEMRA) cells was already high in young children and did not increase further with age, possibly reflecting high levels of antigen exposure in early life. A decline in absolute numbers of all myeloid and lymphoid cell populations was observed throughout life (supplemental Tables 2 and 3A-B).

Phenotypic differentiation of NK cells is biphasic and is most rapid during the first 5 years of life

We identified 3 distinct populations of $CD56^{dim}$ NK cells: $CD57^{-}$, $CD57^{+}$, and those with intermediate CD57 expression ($CD57^{int}$)[6] (Figure 2A). The proportion of $CD57^{-}$ $CD56^{dim}$ NK cells declined significantly with age, mirrored by increasing proportions of $CD57^{+}$ NK cells; the proportion of $CD57^{int}$ cells was stable, consistent with this being a transitional population (Figure 2B). Strikingly, this was a biphasic rather than a linear process, with the most marked changes in CD57 subset distribution occurring in children aged 5 years or younger, with very little change in subset distribution after the age of 10 years (supplemental Figure 3A-C).

The frequency of NK cells expressing CD94, which partners both NKG2A and NKG2C at the cell surface, remained stable throughout life, suggesting that the proportion of NK cells expressing either NKG2A or NKG2C also remains stable (Figure 2C-F; supplemental Figure 3D). However, within the $CD94^{+}$ population, the proportion of $NKG2A^{+}$ cells decreased with increasing age (Figure 2E; $P = .03$, analysis of variance), whereas the proportion of $NKG2C^{+}$ cells increased (Figure 2F; $P = .02$, analysis of variance). Increasing proportions of $NKG2C^{+}$ NK cells were offset by decreasing NK cell numbers, such that the absolute number of $NKG2C^{+}$ cells remained stable throughout life (supplemental Table 1).

We then assessed whether changes in CD57 expression mirrored changes in NKG2A/NKG2C expression (Figure 3). The proportion of $CD57^{-}$ cells within the $NKG2A^{+}$ subset decreased significantly with increasing age, with a reciprocal enrichment of $CD57^{int}$ and $CD57^{+}$ NK cells (Figure 3A). Nevertheless, the majority of $NKG2A^{+}$ NK cells remained $CD57^{-}$, even in older individuals (Figure 3A). In contrast, $NKG2C^{+}$ NK cells are frequently $CD57^{+}$ even in children younger than 2 years, and the majority of $NKG2C^{+}$ NK cells are $CD57^{+}$ by the age of 5 years (Figure 3B). The mean fluorescence intensity (MFI) of CD57 expression was very low on $NKG2A^{+}$ NK cells (at all ages) but increased significantly with increasing age on $NKG2C^{+}$ cells (Figure 3C; supplemental Figure 3G-H), suggesting that $NKG2C^{+}$ NK cells differentiate rapidly in this cohort (gaining full CD57 expression very early in life), whereas $NKG2A^{+}$ NK cells differentiate only very slowly. This rapid expansion and differentiation of the $NKG2C^{+}$ NK cell population is likely a consequence of perinatal HCMV infection. Moreover, anti-HCMV IgG titer was negatively correlated with the frequency of $CD57^{+}$ NK cells (supplemental Figure 4), suggesting that advanced NK cell differentiation may be associated with control of HCMV or vice versa. EBV serostatus, which has been associated with altered NK cell phenotype in HCMV-exposed Europeans,[7] had no significant effect on NK cell subset distribution, other than a minor increase in $CD56^{dim}$ cell frequency (supplemental Figure 5A-G), supporting a recent paper suggesting that acute EBV coinfection has no major effect on $NKG2C^{+}CD57^{+}$ NK cells.[30]

Rapid functional maturation of NK cells during childhood in The Gambia

To assess the functional consequences of these phenotypic changes, PBMCs were cultured in vitro with K562 target cells or with high concentrations of cytokines (IL-12 and IL-18; HCC); NK cell degranulation (CD107a), CD25, and IFN-γ expression were assessed by flow cytometry (Figure 4). Spontaneous low-level degranulation and IFN-γ production was observed among unstimulated cells from children younger than 10 years, perhaps indicating in vivo NK cell activation (Figure 4C,I). Incubation with K562 cells increased NK cell degranulation, but this did not differ with age (Figure 4D). Conversely, degranulation and upregulation of CD25 and IFN-γ production in response to HCC (Figure 4E,H,K) were both strongly age-related, being

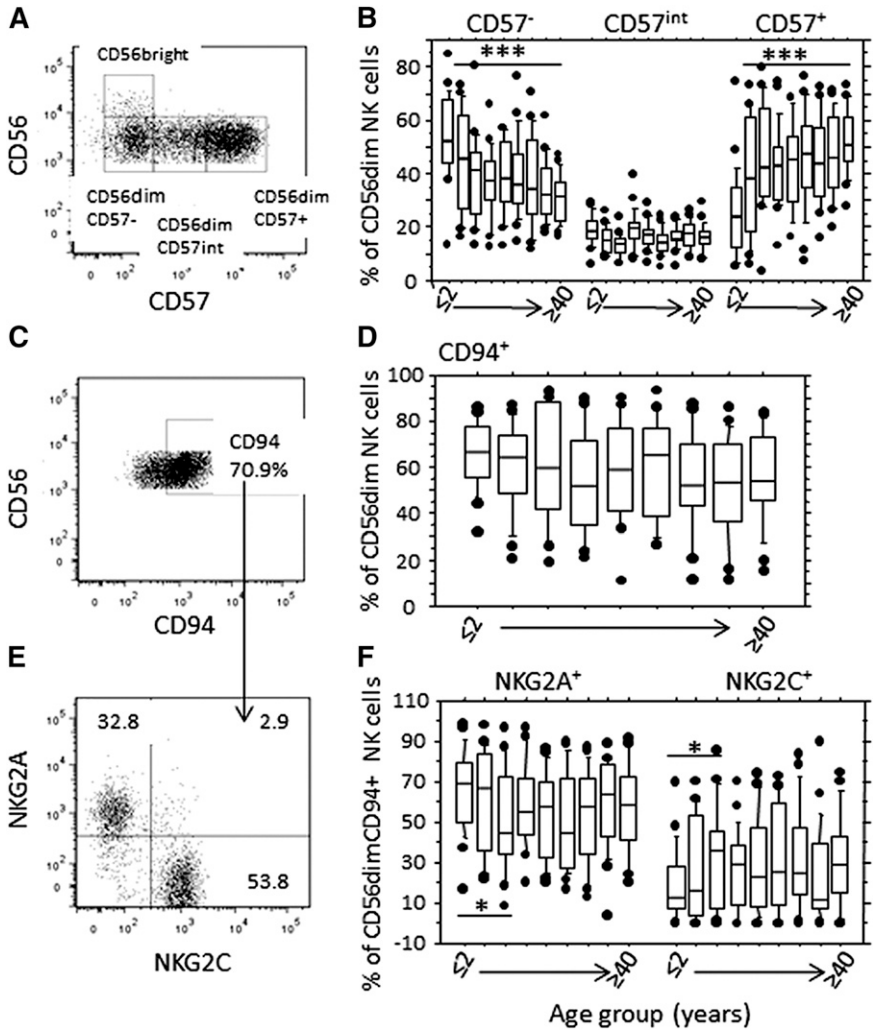

Figure 2. Age-related changes in frequencies of CD57- and c-type lectin-like receptor- expressing NK cell subsets (A). $CD56^{dim}$ cells were gated into $CD57^{-}$, $CD57^{intermediate}$, and $CD57^{+}$ subsets. The $CD57^{-}$ population was gated using an isotype-matched control reagent, and the $CD57^{+}$ gate was set at an MFI of 3000. (B) Frequency distribution by age group of $CD57^{-}$, $CD57^{int}$, and $CD57^{+}$ subsets within the $CD56^{dim}$ NK cell population. Asterisks denote statistically significant trends for changes in NK cell subset frequency by age (***$P < .001$, *F*-test). (C) Gating strategy for $CD94^{+}$ cells and (E) CD94 $NKG2A^{+}$ and $CD94^{+}NKG2C^{+}$ cells within the $CD56^{dim}$ NK cell subset. (D) Frequencies of $CD94^{+}$ and (F) $NKG2A^{+}$, and $NKG2C^{+}$ NK cells by age group. Asterisks denote statistically significant differences in frequencies of $NKG2A^{+}$ and $NKG2C^{+}$ cells by age group (*$P < .05$, analysis of variance). Horizontal bars represent median values, boxes extend from the 25th to the 75th percentile, and whiskers represent the 95th percentiles. Age groups are as shown in Figure 1.

significantly higher in children younger than 10 years than in older individuals (supplemental Figure 6).

Spontaneous NK cell degranulation could be attributed to $CD56^{dim}CD57^{-}$ cells (Figure 5A), whereas spontaneous expression of CD25 and IFN-γ production were restricted to the $CD56^{bright}$ subset (Figure 5B-C). CD107a and CD25 expression were observed in all NK cell subsets after incubation with K562 cells. Although this did not vary with age, it was higher in $CD57^{-}$ cells than in $CD57^{int}$ and $CD57^{+}$ cells (Figure 5D-E), consistent with patterns of expression of the NKp30 activating receptor (which binds B7-H6 on K562 cells).[31,32] As expected, K562 cells induced little IFN-γ secretion from any NK cell subset (Figure 5F).

$CD57^{-}$ NK cells (but not $CD57^{int}$ or $CD57^{+}$ cells) degranulated extensively in response to cytokine stimulation (Figure 5G), and cytokine-induced CD25 expression and IFN-γ production declined with progressive NK cell differentiation, being highest in the $CD56^{bright}$ subset and lowest in the $CD56^{dim}$ $CD57^{+}$ subset (Figure 5H-I). Although there was a trend for increasing CD107a and CD25 expression with increasing age in $CD57^{int}$ and $CD57^{+}$ NK cells after cytokine stimulation (Figure 5G-H), this was only significant when comparing the very youngest and very oldest age groups ($P < .01$, analysis of variance).

Thus, although subtle age-associated changes in NK cell function may be evident within subsets, changing NK cell function with age is primarily a result of the changing proportion of cells within subsets.

Effect of *NKG2C* genotype on NK cell numbers and phenotype

Lack of NKG2C expression because of deletion of the *NKG2C* locus has been reported in several populations.[27,33-35] Nineteen of 181 individuals tested here (10.5%) were $NKG2C^{-/-}$ (and lacked surface expression of NKG2C), whereas 68 individuals (37.6%) were heterozygotes, giving a haplotype frequency of 29.3%. $NKG2C^{-/-}$ individuals were distributed evenly across age groups and between the sexes (Table 1).

NKG2C genotype did not affect frequencies of total, $CD56^{bright}$, or $CD56^{dim}$ NK cells, although, consistent with published data,[34] $NKG2C^{-/-}$ children younger than 10 years had lower absolute numbers of NK cells when compared with $NKG2C^{+/-}$ children (supplemental Figure 7). However, $NKG2C^{-/-}$ individuals had significantly lower frequencies of $CD56^{dim}$ $CD94^{+}$ NK cells than did $NKG2C^{+/-}$ and $NKG2C^{+/+}$ individuals (Figure 6A). Absolute numbers of $NKG2A^{+}$ cells were unaffected by genotype (supplemental Figure 8B), whereas absolute numbers of $CD94^{+}$ cells were significantly lower among $NKG2C^{-/-}$ individuals (supplemental Figure 8A). This is consistent with $CD94^{+}NKG2A^{+}$ to $CD94^{+}NKG2C^{+}$ ratios being determined by expansion within the $CD94^{+}NKG2C^{+}$ subset, rather than by conversion of $NKG2A^{+}$

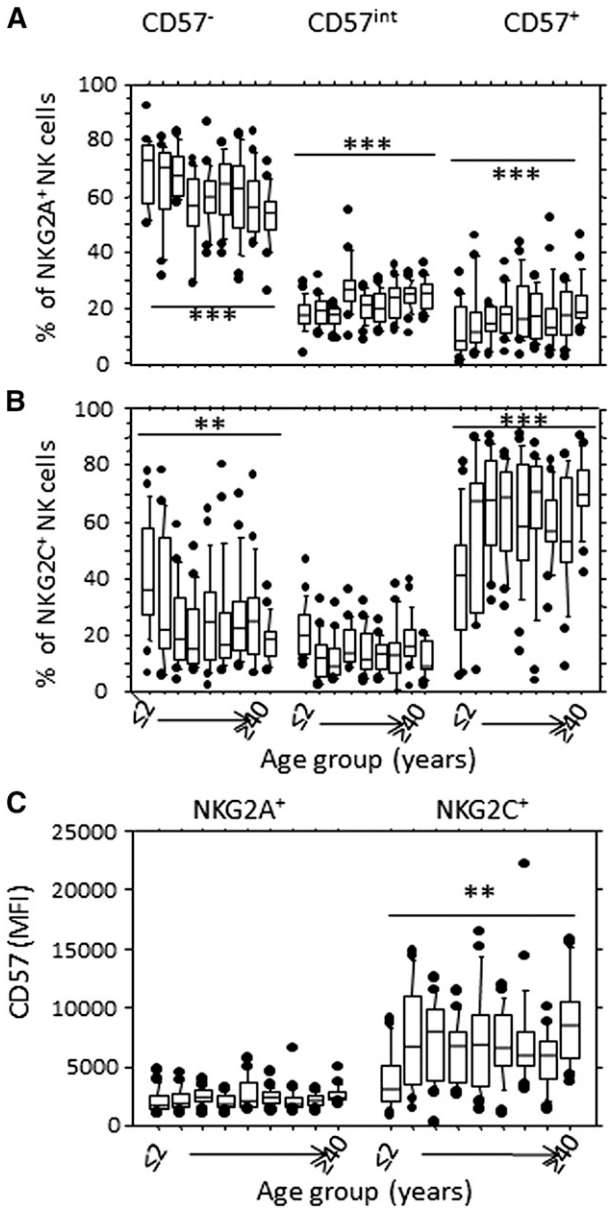

Figure 3. CD57 is preferentially expressed on NKG2C$^+$ NK cells. CD56^{dim} NK cells were gated as in Figure 1A, and the frequency of CD57$^-$, CD57^{int}, and CD57$^+$ cells is shown within (A) CD94/NKG2A$^+$ or (B) CD94/NKG2C$^+$ NK cells, by age group. (C) MFI for CD57 expression on NKG2A$^+$ and NKG2C$^+$ NK cells by age group. Horizontal bars represent median values, boxes extend from the 25th to the 75th percentile, and whiskers represent the 95th percentiles. Asterisks denote statistically significant trends by age within each subset (**$P < .01$; ***$P < .001$, *F*-test). Age groups are as shown in Figure 1.

cells to NKG2C$^+$ cells. A significant gene dosage effect was observed, with *NKG2C*$^{+/-}$ individuals having intermediate frequencies (Figure 6B) and numbers (supplemental Figure 8) of CD94$^+$NKG2A$^+$ and CD94$^+$NKG2C$^+$ cells. A modest decrease in the MFI for NKG2C expression was observed in *NKG2C*$^{+/-}$ compared with *NKG2C*$^{+/+}$ individuals, although this did not reach statistical significance (supplemental Figure 9). *NKG2C*$^{-/-}$ children (younger than 10 years) had significantly lower frequencies of CD57$^+$ NK cells than did heterozygous and homozygous *NKG2C*$^+$ children, with a reciprocal increase in both CD57$^-$ and CD57^{int} NK cells (Figure 6C). This effect was absent in individuals older than 10 years.

Finally, to explore whether the *NKG2C* genotype might affect control of HCMV, we examined the relationships among age, genotype, and anti-HCMV antibody titer (Figure 6D). Anti-HCMV antibody titers were markedly and significantly higher in *NKG2C*$^{-/-}$ than in NKG2C$^{+/+}$ children (younger than 10 years) (Figure 6D), suggesting that inferior control of HCMV infections in these children may lead to more frequent reactivation and boosting of antibody responses. This effect was not observed in older individuals and appeared to be specific for HCMV, as no effect of *NKG2C* genotype was observed on titers of antibodies to childhood vaccine antigens or EBV (supplemental Figure 10). One explanation for this is that lack of NKG2C$^+$ NK cells may hinder control of HCMV, such that the ability to control HCMV viral load (as reflected by anti-HCMV titer) develops more slowly in children who lack NKG2C.

Discussion

It is increasingly appreciated that NK cells are genetically, phenotypically, and functionally diverse, both at the human population level[36] and within individuals.[37] Moreover, NK cells differentiate through the life course, reflecting the interplay of genes and environment. These adaptations substantially modify NK cell function[20,38,39] and are beginning to be associated with health outcomes.[9] Age is a major determinant of NK cell phenotype and function,[18-22] but it is not yet clear whether this is a result of primary, age-intrinsic processes or whether age is simply a marker for cumulative environmental exposures. HCMV infection is a major confounder of the association between age and NK cell function,[11,26,40] but HCMV status is not reported in many published studies, hindering data interpretation. To unpick these issues, detailed phenotypic and functional studies are required across the entire age span and among genetically diverse populations in different environments. The data presented here represent the most comprehensive study to date of NK cell phenotype and function from infancy to mature adulthood and the first such study in an African community and in a population where confounding by HCMV infection status is minimized because of near-universal HCMV infection in infancy.

We previously identified an apparently transitional population of CD56^{dim} NK cells with intermediate CD57 expression (CD57^{int}), expressing intermediate levels of CD16, CD62L, IL-12R, and IL-18R and with a capacity for degranulation, CD25 expression, and IFN-γ production between CD57$^-$ and CD57$^+$ NK cells.[6] Here we observe that although frequencies of CD57$^-$ cells decrease and frequencies of CD57$^+$ cells increase with age, a small but persistent population of CD57^{int} NK cells is present at all ages, suggesting that differentiation of CD57$^-$ to CD57$^+$ NK cells occurs at a similar rate throughout the life course. If so, age-related changes in CD57$^-$ and CD57$^+$ NK cell frequencies must reflect differential rates of loss or proliferation of these 2 subsets, rather than changing rates of cell conversion. Rates of both apoptosis and proliferation are reportedly very high in human NK cells,[41] but whether these rates differ between CD57$^-$ and CD57$^+$ NK cells is unknown.

One striking observation in this population is the very high frequency of fully differentiated CD56^{dim}NKG2C$^+$CD57$^+$ NK cells in very young children; these cells represent up to 50% of all NK cells in 1- to 2-year-olds and up 80% of cells in 6- to 9-year-olds, with the mature adult range (~30% to 70%) being reached by the age of 10 years. In Europeans, proportions of CD57$^+$ NK cells range from zero at birth (cord blood) to median values of ~50% in adults,[20] with values being higher in HCMV$^+$ individuals (30%-70%) than in

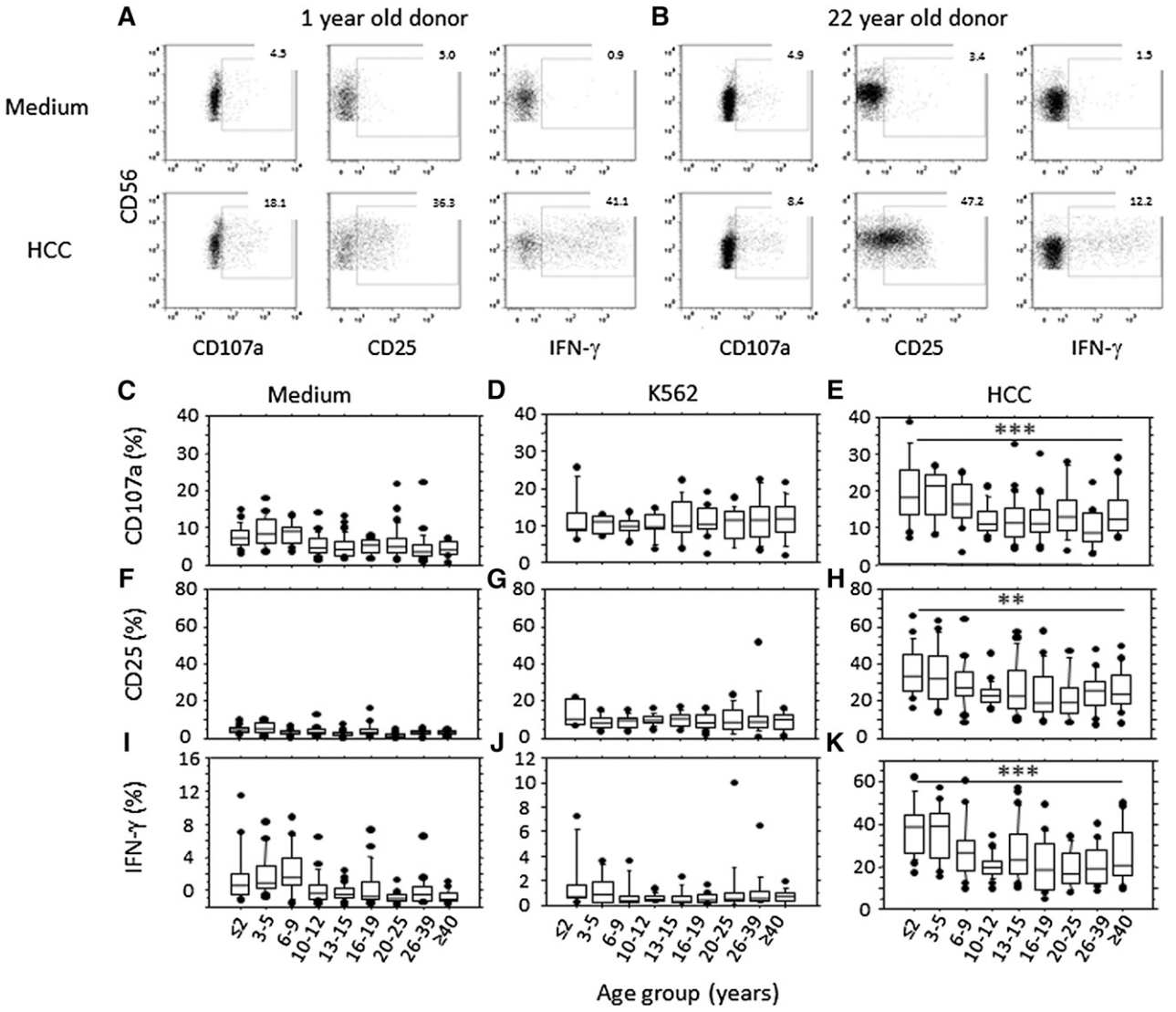

Figure 4. Age-associated changes in NK cell function. Example flow cytometry plots are shown for CD3$^-$ lymphocytes from a 1-year-old (A) and a 22-year-old (B), cultured in medium alone (*top*) or stimulated with high concentrations of IL-12+ IL-18 (HCC, *bottom*) and assayed for degranulation (CD107a), CD25, and IFN-γ expression. (C-K) NK cells were assayed for degranulation (C-E), CD25 (F-H), or IFN-γ (I-K) expression after in vitro culture in medium alone (C,F,I) or with K562 target cells (D,G,J) or IL-12 + IL-18 (HCC; E,H,K). Horizontal bars represent median values, boxes extend from the 25th to the 75th percentile, and whiskers represent the 95th percentiles. Asterisks denote significant age-related trends for frequencies of NK cells expressing CD107a, CD25, or IFN-γ (**$P < .01$;*** $P < .001$, *F*-test).

HCMV$^-$ subjects (25%-50%).[42] Although we could not compare our data with a fully age-matched, low-HCMV prevalence cohort, the frequency of CD57$^+$ NK cells in HCMV-seropositive adult Gambians is significantly higher than in an age-matched HCMV-seropositive UK cohort, confirming more rapid or extensive NK cell differentiation among Gambians (supplemental Figure 11). This may reflect either HCMV infection much earlier in life in The Gambia or a higher prevalence of other infections that further expand the NKG2C$^+$CD57$^+$ NK cell population in HCMV$^+$ individuals.[43] Data on HCMV$^+$ and HCMV$^-$ European children are needed to confirm this.

Interestingly, HCMV and EBV coinfection did not affect NK cell phenotype or function. EBV coinfection has been associated with more extensive NK cell differentiation compared with HCMV alone in some European studies,[7] but not in a recent US study,[30] suggesting that perinatal HCMV infection alone is sufficient to drive NK cell differentiation or that infections other than EBV may also have an effect in this Gambian cohort. Of note, the biphasic kinetic of NK cell differentiation is not accompanied by a similar biphasic differentiation of T-cell populations, which is consistent with the suggestion that HCMV infection independently affects T-cell and NK cell populations.[44]

Age-related differences in NK cell function were entirely a result of differences in the proportions of CD57$^-$ and CD57$^+$ NK cells. In Caucasian adults, cytokine-induced degranulation, CD25 expression, and IFN-γ production all decline with increasing levels of CD57 expression,[2,4-6] in parallel with reduced expression of IL-12 and IL-18 receptors,[6] whereas CD57 expression has much less effect on responses receptor crosslinking.[6] This association between CD57 expression and NK cell function also holds true in The Gambia and in children as young as 3 to 5 years of age. Because CD57 expression is, to a large extent, driven by HCMV, it appears that infection with HCMV very early in life rapidly skews the entire NK cell population to missing/altered-self/antibody-dependent cytotoxicity at the expense of cytokine-driven responses.[6,45] This skewing of NK cell function is much more marked among Gambian adults than among

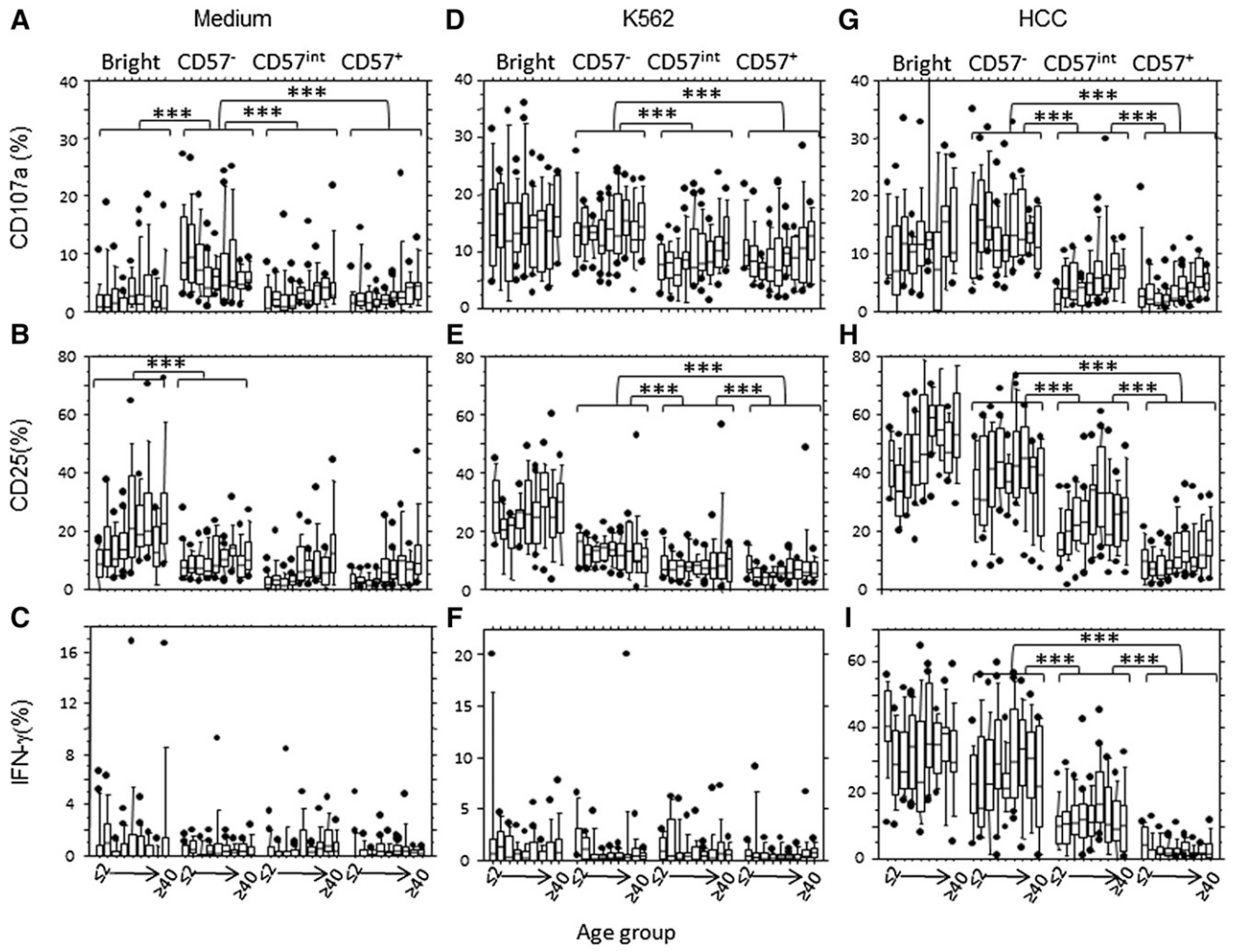

Figure 5. NK cell function reflects CD57 expression, irrespective of age. Bright ($CD56^{bright}CD57^{-}$), $CD57^{-}$ ($CD56^{dim}CD57^{-}$),CD57int ($CD56^{dim}CD57^{int}$) and $CD57^{+}$ ($CD56^{dim}CD57^{+}$) NK cell subsets were analyzed for CD107a (A,D,G), CD25 (B,E,H), or IFN-γ (C,F,I) after in vitro culture in medium alone (A-C), with K562 target cells (D-F) or with IL-12 + IL-18 (HCC; G-I). Horizontal bars represent median values, boxes extend from the 25th to the 75th percentile, and whiskers represent the 95th percentiles. There were no significant age-related trends in response within any of the subsets. Asterisks denote statistically significant differences between $CD57^{-}$, $CD57^{int}$, and $CD57^{+}$subsets ($P < .001$ for all comparisons, Wilcoxon-signed rank).

age-matched HCMV seropositive UK adults, again presumably reflecting an earlier age of HCMV infection or increased prevalence of coinfections in The Gambia (supplemental Figure 11). Altered NK cell function so early in life could contribute to associations among perinatal HCMV infection, slower growth, and increased rates of hospitalization, as observed in Zambian children.[46]

In line with the near-universal HCMV infection in infancy in our cohort and the well-documented expansion of $CD57^{+}NKG2C^{+}$ NK cells in $HCMV^{+}$ individuals,[11] frequencies of $NKG2C^{+}$ cells were high in all age groups. Frequencies of $NKG2C^{+}$ NK cells were lower in very young children than in older age groups, but adult frequencies were achieved by the age of 6 to 9 years, suggesting that expansion of the $NKG2C^{+}$ subset begins very quickly after HCMV infection and may continue for some years. This is consistent with data from transplant recipients with acute HCMV infection or HCMV reactivation, where frequencies of $NKG2C^{+}$ NK cells increase within a month of infection/reactivation and continue to increase for at least 12 months,[14,26,40] and with reports of significantly higher frequencies of $NKG2C^{+}$ NK cells in $HCMV^{+}$ compared with $HCMV^{-}$ children younger than 2 years.[47]

In Caucasian adults, the $NKG2C^{+}$ NK cells induced by HCMV infection tend to coexpress CD57.[14] This was also the case here, although both the frequency of $NKG2C^{+}$ cells expressing CD57 and the median MFI of CD57 expression were lower in children younger than 2 years than in older individuals. In contrast, CD57 is expressed only at low intensity on $NKG2A^{+}$ NK cells at all ages. These observations are consistent with a model in which peptides from HCMV UL40 bind to HLA-E, stabilizing it at the surface of infected cells, where it drives activation, proliferation, and differentiation (including expression of CD57) of NK cells expressing NKG2C (the activating receptor on NK cells for HLA-E) while simultaneously inhibiting proliferation and differentiation of cells expressing NKG2A (the inhibitory HLA-E receptor).[10,48-50]

Although CD94/NKG2C and CD94/NKG2A are not the only NK cell receptors for HCMV,[51-53] lack of *NKG2C* was clearly linked to delayed NK cell differentiation and maturation. $NKG2C^{+/-}$ heterozygotes had lower frequencies of $NKG2C^{+}$ NK cells than did $NKG2C^{+/+}$ individuals (consistent with a previous report[33]), and the frequency and absolute number of $CD94^{+}$ cells was positively associated with *NKG2C* copy number, consistent with the hypothesis that $NKG2C^{+}$ NK cell numbers expand by proliferation, rather than by transformation from $NKG2A^{+}$ cells. Importantly, however, a high proportion of $CD56^{dim}$ NK cells in $NKG2C^{-/-}$ individuals lack expression of CD94/ NKG2A, as well as CD94/NKG2C, raising

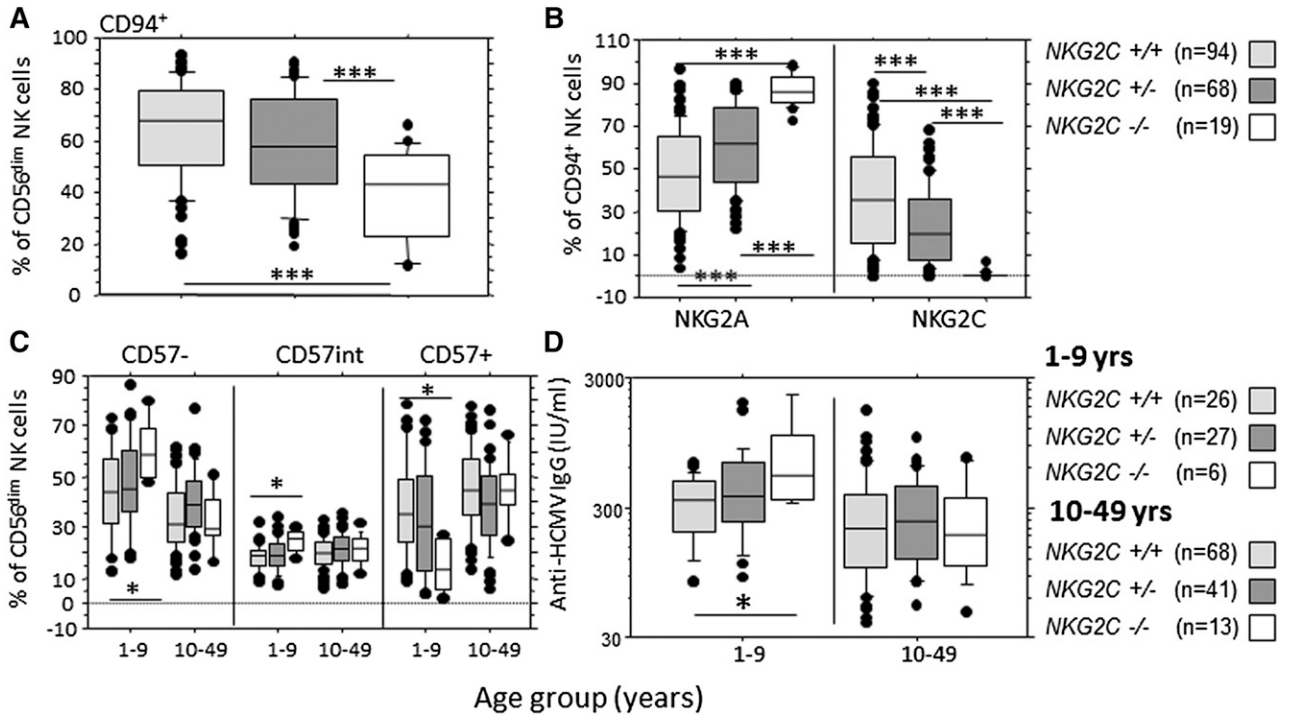

Figure 6. Effect of *NKG2C* genotype on NK cell maturation phenotype and HCMV antibody titer. (A) Frequency of $CD94^+$ cells within the $CD56^{dim}$ NK cell population in individuals with zero ($NKG2C^{-/-}$), 1 ($NKG2C^{+/-}$), or 2 ($NKG2C^{+/+}$) copies of the *NKG2C* gene. (B) Effect of *NKG2C* genotype on the frequencies of $CD94^+$ NK cells expressing either $NKG2A^+(NKG2C^-)$ or $NKG2C^+(NKG2A^-)$ cells. (C) Effect of *NKG2C* genotype on the frequency of $CD57^-$, $CD57^{int}$, and $CD57^+$ NK cells in subjects younger than 10 and 10 or more years of age. (D) Anti-HCMV antibody titers by age (years) and $NKG2C^-$genotype. Horizontal bars represent median values, boxes extend from the 25th to the 75th percentile, and whiskers represent the 95th percentiles. Asterisks denote statistically significant differences between genotypes for all comparisons shown ($^*P < .05$, $^{***}P < .001$, analysis of variance).

questions about which other receptors might be expressed on these cells to maintain NK cell homeostasis and HCMV latency. HCMV reactivation in recipients of $NKG2C^{-/-}$ stem cells drives differentiation of functional KIR+NKG2A- NK cells,[53] suggesting that activating KIR may compensate for lack of CD94/NKG2C. Stable expansions of KIR^+ $NKG2A^-NKG2C^-$ NK cells have also been observed in HCMV-seropositive adults.[54]

Consistent with activation and expansion of $NKG2C^+$ cells before their acquisition of CD57, proportions of $CD57^+$ NK cells were significantly lower in $NKG2C^{-/-}$ subjects than in those with 1 or more copies of *NKG2C*, and in particular for children younger than 10 years. The magnitude of this effect is remarkable, achieving statistical significance despite the rather small number of $NKG2C^{-/-}$ subjects, and is likely to be highly biologically relevant. It would be interesting to know whether delayed NK cell differentiation in HCMV-infected $NKG2C^{-/-}$ subjects is seen in other populations and whether it confers any survival advantage or whether this is offset by impaired control of HCMV (as implied by the significantly higher anti-HCMV antibody titers). These studies will need to be large enough to achieve statistical power, which will depend on both the prevalence of the *NKG2C*-null haplotype and of HCMV. The 29.3% haplotype frequency of the *NKG2C* deletion in our African cohort is higher than that recorded elsewhere.[33,35,55] Whether the frequency of this haplotype is linked to current or historic intensities of HCMV infection might also merit further investigation.

Despite our study cohort being almost uniformly HCMV-seropositive, considerable heterogeneity is observed in NK cell phenotype and function within each age group. Although some of this is heterogeneity may be genetically determined and/or stochastic,[37] exposure to infections in addition to HCMV may also affect NK cell maturation.[7,15,17,56] Further studies are needed to determine whether this is simply a cytokine-driven expansion, and thus likely to occur in response to many acute inflammatory stimuli, or whether some pathogens express specific ligands for $CD57^+$ $NKG2C^+$ NK cells.

In summary, our study has revealed rapid phenotypic and functional differentiation of peripheral NK cells in a population with extremely high rates of perinatal HCMV infection. Intriguingly, NK cell phenotype seems to be highly dependent on the expression of NKG2C, reaffirming the notion that signaling via NKG2C is causally linked to NK cell differentiation. Further studies are now warranted to evaluate the effect of early HCMV infection on the ability of NK cells to contribute to protection from other infections throughout the life course.

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Authorship

Contribution: M.R.G. designed research, performed experiments, analyzed and interpreted data, and wrote the manuscript; M.J.W. designed and performed research and analyzed and interpreted data; A.D. designed and performed research; C.M.N. performed experiments and analyzed data; A.G. performed research; C.B. analyzed data; S.E.M. designed research and coordinated recruitment of study subjects; and E.M.R. designed research, supervised data collection, directed data analysis, and wrote the manuscript.

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Rapid NK cell differentiation in a population with near-universal human cytomegalovirus infection is attenuated by NKG2C deletions

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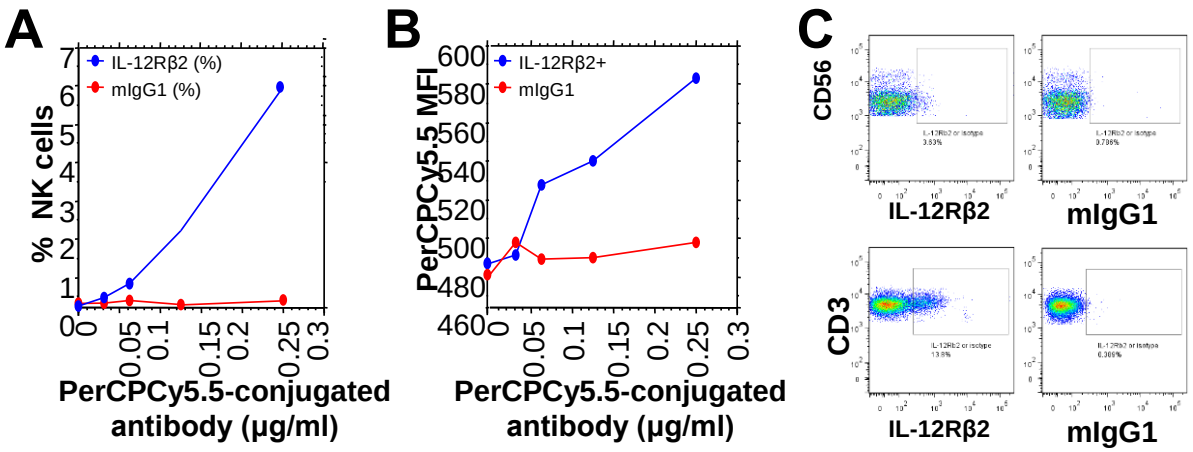

Appendix IX. Titration and isotype control staining of PerCPCy5.5-conjugated anti-IL-12Rβ2[1]. Purified NK cells (>95%) were cultured for 18 hours with a high concentration of IL-12 (5mg/ml) and IL-18 (50ng/ml) and stained with increasing concentrations of PerCPCy5.5-conjugated IL-12Rβ2, or a monoclonal IgG isotype control (mIgG1; mouse anti-human CD19), as labelled. The percentage of NK cells positive for PerCPCy5.5 **(A)** and the MFI of these PerCPCy5.5+ cells **(B)** were compared between NK cell stained with anti-IL-12Rβ2 and mIgG1 antibodies. Resting PBMC were then stained *ex vivo* with anti-IL-12Rβ2 or mIgG1 isotype control and expression on NK and T cells were analysed **(C)**.

[1] Conjugation and titration of IL-12Rβ2 antibody were performed by Martin Goodier.

Impaired NK Cell Responses to Pertussis and H1N1 Influenza Vaccine Antigens in Human Cytomegalovirus-Infected Individuals

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NK cells contribute to postvaccination immune responses after activation by IL-2 from Ag-specific memory T cells or by cross-linking of the low-affinity IgG receptor, CD16, by Ag–Ab immune complexes. Sensitivity of NK cells to these signals from the adaptive immune system is heterogeneous and influenced by their stage of differentiation. $CD56^{dim}CD57^{+}$ NK cells are less responsive to IL-2 and produce less IFN-γ in response to T cell–mediated activation than do $CD56^{bright}$ or $CD56^{dim}CD57^{-}$ NK cells. Conversely, NK cell cytotoxicity, as measured by degranulation, is maintained across the $CD56^{dim}$ subsets. Human CMV (HCMV), a highly prevalent herpes virus causing lifelong, usually latent, infections, drives the expansion of the $CD56^{dim}CD57^{+}$ $NKG2C^{+}$ NK cell population, skewing the NK cell repertoire in favor of cytotoxic responses at the expense of cytokine-driven responses. We hypothesized, therefore, that HCMV seropositivity would be associated with altered NK cell responses to vaccine Ags. In a cross-sectional study of 152 U.K. adults, with HCMV seroprevalence rate of 36%, we find that HCMV seropositivity is associated with lower NK cell IFN-γ production and degranulation after in vitro restimulation with pertussis or H1N1 influenza vaccine Ags. Higher expression of CD57/NKG2C and lower expression of IL-18Rα on NK cells from HCMV seropositive subjects do not fully explain these impaired responses, which are likely the result of multiple receptor–ligand interactions. This study demonstrates for the first time, to our knowledge, that HCMV serostatus influences NK cell contributions to adaptive immunity and raises important questions regarding the impact of HCMV infection on vaccine efficacy. ***The Journal of Immunology*****, 2015, 194: 4657–4667.**

Natural killer cells are traditionally classified as cells of the innate immune system but can also act as mediators of adaptive immunity. In addition to their well-recognized role in Ab-dependent cytotoxicity (ADCC), recent research has demonstrated a potential contribution to adaptive responses through their activation by Ag-specific $CD4^{+}$ T cell–derived IL-2 (1–7). The heightened IFN-γ response of NK cells in the context of a vaccine recall response suggests that NK cells may play a role in protection from vaccine-preventable diseases, particularly as NK cells respond more quickly than T cells and comprise as much as 70% of all IFN-γ–producing cells in the first 12–24 h of the recall response (3).

We have shown, using the individual components of the diphtheria toxoid/tetanus toxoid/whole-cell pertussis vaccine, that activation of NK cells after restimulation with vaccine Ags is heterogeneous, with $CD56^{bright}$ and $CD56^{dim}CD57^{-}$ NK cells being most responsive as measured by surface expression of the high-affinity IL-2 receptor (CD25) and accumulation of intracellular IFN-γ ($CD25^{+}$IFN-γ^{+}) (6). Expression of CD57 by $CD56^{dim}$ NK cells was associated with a reduced capacity to produce IFN-γ, although degranulation responses were maintained (6). These data are consistent with the accepted model of NK cell maturation whereby acquisition of CD57 is a marker of decreased sensitivity to exogenous cytokine stimulation (8, 9).

Human CMV (HCMV) infection drives profound changes in the NK cell repertoire. In particular, HCMV infection is strongly associated with preferential expansion of the $CD56^{dim}CD57^{+}$ $NKG2C^{+}$ NK cell subset (10–12). This has direct implications for NK cell function as $CD56^{dim}CD57^{+}NKG2C^{+}$ NK cells degranulate and secrete cytokines such as IFN-γ and TNF-α in response to cross-linking of CD16 (by IgG) or natural cytotoxicity receptors (by infected, stressed, or transformed cells) but respond poorly to proinflammatory cytokines such as IL-12 and IL-18 (12, 13).

These observations imply that, in the context of infection or vaccination, NK cells from HCMV-seropositive ($HCMV^{+}$) individuals may effectively mediate ADCC after cross-linking of CD16 by IgG in immune complexes (11, 13, 14), but may respond poorly to inflammatory cytokines (reviewed in Ref. 15). Specifically, the expanded $CD56^{dim}CD57^{+}NKG2C^{+}$ NK cell subset may be less sensitive to IL-2 produced by Ag-specific $CD4^{+}$ T cells and IL-12/IL-18 from accessory cells, such as dendritic cells and macrophages (3, 6). However, much of the data on skewing of the NK cell repertoire in $HCMV^{+}$ individuals comes from studies of

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The online version of this article contains supplemental material.

Abbreviations used in this article: ADCC, Ab-dependent cytotoxicity; AEU, arbitrary ELISA unit; HCC, high concentration of cytokines; HCMV, human CMV; LCC, low concentration of cytokines; MFI, median fluorescence intensity; PT, pertussis toxin.

hematopoietic stem cell or solid organ transplantation (11, 16, 17), and follow-up of these patients over time, in terms of susceptibility to infection or response to vaccination, is lacking. As a result, the true functional significance of HCMV-driven NK cell phenotypic changes is poorly understood. Moreover, previous investigations of the impact of HCMV infection on vaccination have produced rather inconsistent results, with some studies reporting impaired vaccine responses in $HCMV^+$ donors (18–23), whereas others find no impact of HCMV infection (24–27). The impact of HCMV-driven immune differentiation on vaccine responsiveness and efficacy is therefore still unclear.

The aim of this study, therefore, is to compare NK cell responses to Ags previously encountered during immunization (*Bordetella pertussis*) or during natural infection (H1N1 influenza virus) in $HCMV^-$ and $HCMV^+$ individuals.

Materials and Methods

Study subjects

Volunteers ($n = 152$) were recruited from staff and students at the London School of Hygiene and Tropical Medicine. All subjects gave written consent and the study was approved by the London School of Hygiene and Tropical Medicine Ethics Committee. Each subject provided a 50-ml venous blood sample, and reported vaccination history was recorded. Subject characteristics are summarized in Table I.

Ab detection by ELISA

Plasma was collected from heparinized whole blood and stored at −80°C until use. HCMV infection status was determined by HCMV IgG ELISA (BioKit). IgG Abs to pertussis toxin (PT; NIBSC) and to formalin-inactivated whole H1N1 influenza virus (influenza A/California/7/2006 (H1N1)v(NYMC-X179A); H1N1; NIBSC) were determined using in-house ELISA assays with goat anti-human IgG-peroxidase (Sigma-Aldrich) as the secondary Ab and SIGMAFAST OPD (Sigma-Aldrich) as the substrate. IgG concentrations were calculated by interpolation from a standard curve, which was produced using anti-pertussis reference serum (NIBSC; IU/ml) or using plasma from a donor with high titers of Abs to H1N1 influenza (IgG concentration expressed in arbitrary ELISA units [AEU]) (28). The pooled AB plasma used for in vitro assays contained 6.8 IU/ml IgG to PT and had an H1N1 IgG titer of 273.8 AEU.

PBMC preparation and culture

PBMCs were isolated from heparinized venous blood on a Ficoll–Hypaque gradient and cryopreserved in liquid nitrogen. Before use, PBMCs were thawed into complete medium (RPMI 1640 supplemented with 100 U/ml penicillin/streptomycin and 20 mM L-glutamine [Life Technologies, Lifesciences] and 10% pooled human AB plasma), washed, and rested for 30 min before use. For some experiments, AB plasma was IgG-depleted using a protein G-Sepharose column (GE Life Sciences).

PBMCs were cultured for 18 h at 37°C at 2×10^5/well in 96-well U-bottom plates (Nunc) in complete medium with or without low concentration of cytokines (LCC; 12.5 pg/ml rhIL-12 [PeproTech] plus 10 ng/ml rhIL-18 [MBL, Woburn, MA]); high concentration of cytokines (HCC; 5 ng/ml rhIL-12 plus 50 ng/ml rhIL-18); rat anti-IL-2 (3 μg/ml; BD Biosciences); rat IgG2A isotype control (3 μg/ml; BD Biosciences; this was included in wells with medium alone, as well as Ag alone); 1 μg/ml formalin-inactivated whole H1N1 influenza virus (NIBSC, as described earlier); 1 IU/ml killed whole-cell *B. pertussis* (pertussis; NIBSC); or MHC class I–deficient K562 target cells (E:T ratio 2:1). GolgiStop (containing Monensin, 1/1500 concentration; BD Biosciences) and GolgiPlug (containing brefeldin A, 1/1000 final concentration; BD Biosciences) were added after 15 h. Anti-CD107a Ab (A488-conjugated; BD Biosciences) was included in the medium for the entirety of cell culture.

For activation via CD16 cross-linking, 96-well flat-bottom plates (Nunc) were coated with anti-human CD16 (BD Biosciences) or an isotype-matched control Ab (mIgG1k; BD Biosciences) overnight at 4°C. Wells were rinsed with PBS before addition of 2×10^5 PBMCs/well, which had been incubated overnight at 37°C with 50 IU/ml IL-2 (PeproTech). Anti–CD107a-A488 Ab was added at the beginning of culture, and cells were harvested after 5 h.

Flow cytometry

PBMCs were stained in 96-well U-bottom plates as described previously (6). In brief, cells were stained with fluorophore-labeled Abs to cell-surface markers, fixed, permeabilized (Cytofix/Cytoperm; BD Biosciences), and stained for intracellular molecules. The following mAbs were used: anti–CD3-V500, anti–CD56-PECy7, anti–IFN-γ–allophycocyanin, anti–CD107a-A488, anti–CD16-allophycocyanin-H7, anti–CD25-allophycocyanin-H7 (all BD Biosciences), anti–CD57-e450, anti–CD25-PerCPCy5.5, anti–CD16-allophycocyanin, anti–CD25-PE, anti–IL-18Rα–PE, anti–IL-18Rα–FITC, anti–IFN-γ–allophycocyanin-e780, anti–CD16–allophycocyanin-e780 (all e-Biosciences), anti–NKG2C-allophycocyanin, anti–NKG2C-PE (both R&D Systems), and anti–NKG2A-FITC (Miltenyi). IL-12Rβ2 Ab was conjugated using EasyLink PE-Cy5 (Abcam). Cells were acquired on an LSRII flow cytometer (BD Biosciences) using FACSDiva software. Data analysis was performed using FlowJo V10 (Tree Star). FACS gates set on unstimulated cells (medium alone or isotype controls) were applied in standard format across all samples and all conditions.

NKG2C genotyping

DNA was extracted from whole blood using a Wizard genomic DNA extraction kit (Promega). Donors were then genotyped for *NKG2C* using touch-down PCR (Phusion High Fidelity PCR kits; New England Biolabs) as described previously (29, 30).

Statistical analyses

Statistical analysis of flow cytometry data was performed using Prism 6 (GraphPad), or STATA/IC 13 (StataCorp), as detailed in the figure legends. Responses where the gated cell subset contained <100 cells were excluded. Mann–Whitney *U* tests were used to compare responses between $HCMV^-$ and $HCMV^+$ donors, and linear regression was used to adjust for sex and age. Unless otherwise stated, statistical tests were one-sided: $****p \leq 0.0001$, $***p < 0.001$, $**p < 0.01$, $*p < 0.05$.

Results

Donor characterization

Subject characteristics are summarized in Table I. Subjects ($n = 152$) ranged in age from 20 to 77 y (median = 33 y). Fifty-five subjects (36.2%) were found to be HCMV seropositive. Anti-HCMV IgG titer increased significantly with increasing age ($R^2 = 0.248$, $p = 0.0001$; Supplemental Fig. 1A), but age did not differ significantly between $HCMV^+$ and $HCMV^-$ donors (two-tailed Mann–Whitney *U* test, $p = 0.561$). Because the proportion of female and male donors differed between the $HCMV^-$ and $HCMV^+$ groups, subsequent analyses were adjusted for sex.

Cells from all 152 subjects were analyzed for responses to pertussis. The median anti-PT IgG titer was higher among $HCMV^-$ donors than among $HCMV^+$ donors, but this difference was not statistically significant (6.7 versus 5.0 IU/ml, two-tailed Mann-Whitney *U* test, $p = 0.078$). One hundred and fourteen donors (75.0%) confirmed that they had been vaccinated against pertussis, but a minority of donors reported that they had not been vaccinated against pertussis ($n = 13$; 8.6%) or were unsure of their vaccination status ($n = 25$; 16.4%). However, the proportions of these individuals did not differ between the $HCMV^+$ and $HCMV^-$ groups, and their Ab titers did not suggest a difference in vaccination history (data not shown).

All donors analyzed for responses to vaccine H1N1 influenza ($n = 52$) confirmed only natural exposure to H1N1, that is, no previous seasonal influenza vaccination. Median anti-H1N1 IgG titers were higher among $HCMV^-$ donors (204.1 AEU/ml) than among $HCMV^+$ donors (187.2 AEU/ml), although this difference was not statistically significant (two-tailed Mann–Whitney *U* test, $p = 0.135$).

Ab and Ag-specific IL-2 drive NK cell responses to pertussis and H1N1 influenza virus

PBMCs from 100 donors were stimulated overnight with pertussis (Fig. 1B–D), and NK cell responses were measured by flow cytometry (Fig. 1A). Significant induction of CD25 and IFN-γ (Fig. 1B and 1C) and degranulation (CD107a; Fig. 1D) was observed in response to pertussis. Analysis of this response by

Table I. Donor characteristics

	HCMV$^-$ (n = 97)	HCMV$^+$ (n = 55)
Median age, y (range)	32 (20–70)	35 (21–77)
Female sex, n (%)	73 (75)	32 (58)
NKG2C genotype $^{+/+}$, $^{+/-}$, $^{-/-}$, n (%)	67/24/2 (72/26/2)	35/17/2 (65/31/4)
NKG2C$^-$ haplotype frequency (%)	15.0	19.4
Median anti-HCMV IgG titer, IU/ml (range)	<0.25	394.2 (31.1–4411.6)
Median anti-PT IgG titer, IU/ml (range)	6.7 (0.5–139.3)	5.0 (0.8–179.9)
Median anti-H1N1 IgG titer, AEUs (range)	214.6 (80.7–953.2)	190.1 (90.2–522.7)

Donors were classified as HCMV$^-$ and HCMV$^+$ by anti-HCMV IgG ELISA, using 0.25 IU/ml as the cutoff per manufacturer's instructions. *NKG2* genotype (*NKG2C*$^{+/+}$, *NKG2C*$^{+/-}$, *NKG2C*$^{-/-}$) was determined by PCR. IgG Ab titers against PT and H1N1 were calculated from interpolation of a reference serum or high-titer donor standard curve, respectively.

CD56^{bright} and CD56^{dim} subsets reveals that the CD56^{dim} cells respond more robustly to pertussis than do the CD56^{bright} NK cells (and are thus the major contributors to the vaccine response; Supplemental Fig. 2A–C).

Coexpression of CD25/IFN-γ was markedly attenuated in the presence of a blocking Ab to IL-2 and after depletion of IgG from the plasma used to supplement the culture medium, indicating a role for both memory T cell–derived IL-2 and Ag-specific Ab in the NK cell IFN-γ response. By contrast, the degranulation response (as measured by cell-surface expression of the lysosomal marker LAMP-1/CD107a) (31) was dependent upon IgG, but not IL-2. The observation that neither anti–IL-2 nor IgG depletion

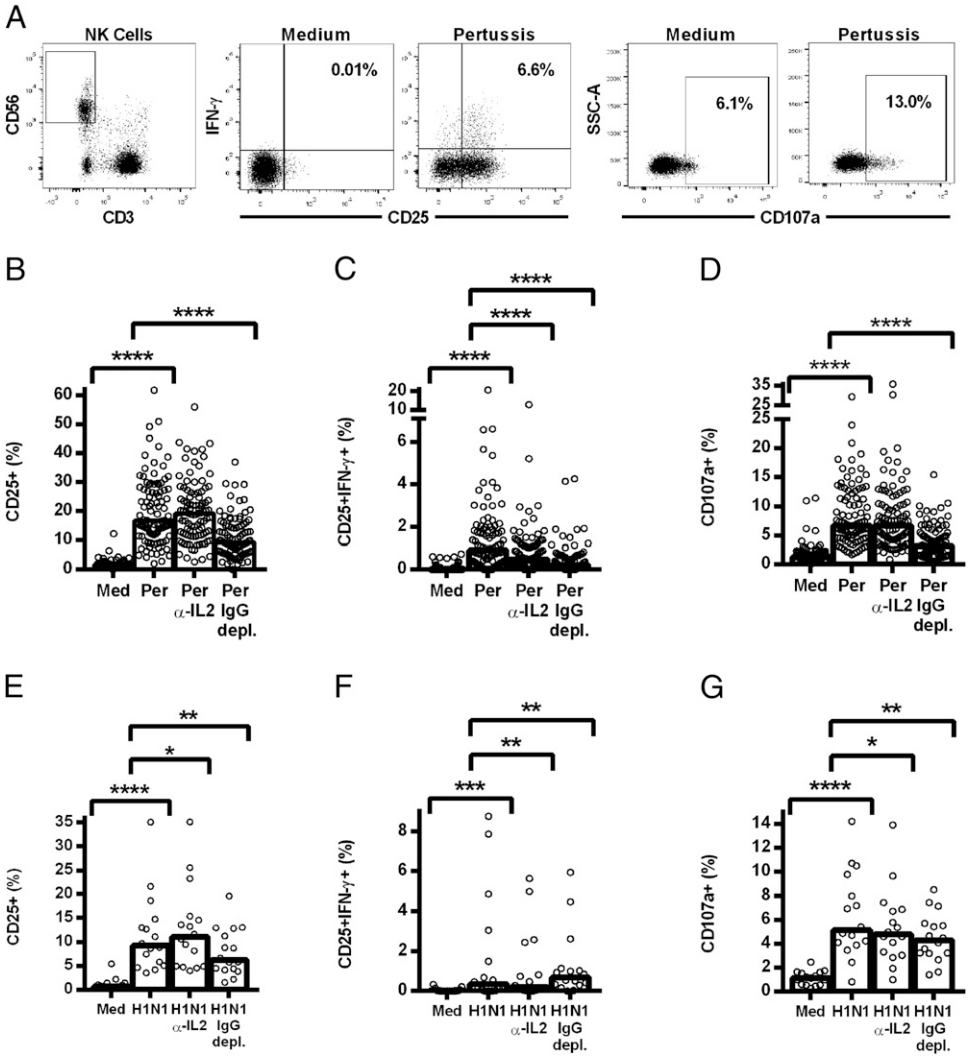

FIGURE 1. NK cell responses to pertussis and H1N1 are inhibited by IL-2 neutralization and IgG depletion. PBMCs were cultured in vitro for 18 h with medium alone, killed whole-cell pertussis (Per), and inactivated whole H1N1 influenza virus (H1N1), pertussis or H1N1 with blocking Ab to IL-2 (Per α-IL-2, H1N1 α-IL-2), or pertussis or H1N1 in IgG-depleted plasma (Per IgG depl., H1N1 IgG depl.). The isotype control Ab (IgG2A) for the IL-2 blocking Ab was included in the medium, pertussis, and H1N1 wells. Representative flow cytometry plots show gating of CD3$^-$CD56$^+$ NK cells and expression of CD25, IFN-γ, and CD107a (**A**). Responses to pertussis (**B**–**D**) and H1N1 (**E**–**G**) were measured by the percentage of NK cells expressing CD25 (B and E), coexpressing CD25/IFN-γ (C and F), and expressing CD107a (D and G). Data were analyzed in Prism using paired, one-tailed Wilcoxon signed-rank tests. Each data point represents one donor, n = 100 (B–D) or n = 16 (E–G), and bar graphs denote medians. ****$p \leq 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

completely abrogated the NK cell IFN-γ response suggests that these two signals may synergize for optimal IFN-γ production.

Cells from a subset of subjects ($n = 16$) were also analyzed for responses to H1N1 influenza in the context of IL-2 blockade or IgG depletion (Fig. 1E–G). As observed with pertussis, statistically significant induction of CD25 (Fig. 1E), CD25/IFN-γ (Fig. 1F), and CD107a (Fig. 1G) was observed in response to restimulation with H1N1 Ag, and IL-2 blocking significantly decreased CD25/IFN-γ expression (Fig. 1F), whereas IgG depletion inhibited the degranulation (CD107a) response (Fig. 1G). Interestingly, and in contrast with the response to pertussis, IgG depletion enhanced IFN-γ production in response to H1N1, and IL-2 blockade slightly decreased degranulation, indicating competition between these pathways for NK cell activation during influenza responses (Fig. 1F).

HCMV infection is associated with impaired NK cell responses to pertussis and H1N1 influenza virus

NK cell responses to pertussis ($n = 152$) and H1N1 ($n = 52$) were compared between $HCMV^-$ and $HCMV^+$ donors (Fig. 2). Consistent with prior observations (3, 6), responses to pertussis and H1N1 were significantly augmented by LCC IL-12 and IL-18 ($p \leq 0.0001$ for all parameters), indicating that in vitro accessory cell activation and production of IL-12 and IL-18 (which is essential for IL-2–mediated NK cell activation) (3, 5, 32) were suboptimal.

Interestingly, in the absence of LCC, pertussis induces stronger NK cell responses than H1NI, whereas in the presence of LCC, H1N1 induces the most robust responses. This may indicate that pertussis induces some IL-12 and IL-18 (such that LCC is redundant in these assays), whereas H1N1 may be a poor inducer of IL-12 and IL-18 but a better inducer of IL-2 or other accessory cytokines. This would be consistent with differences in TLR signaling by RNA viruses such as influenza (TLR3) and Gram-negative bacteria such as pertussis (TLR4) (33–36).

NK cells from both $HCMV^+$ and $HCMV^-$ donors responded to pertussis and H1N1 (with or without LCC; Fig. 2); however, NK cell responses to these two vaccines (whether defined as $CD25^+$, $CD25^+IFN\text{-}\gamma^+$, or $CD107a^+$) were significantly lower among $HCMV^+$ donors than among $HCMV^-$ donors (Fig. 2A and 2B). This was true for both vaccines and all parameters when cells were cultured with LCC, and was also true for the $CD25^+$ and $CD25^+IFN\text{-}\gamma^+$ responses to H1N1 and the $CD25^+$ and $CD107a^+$ responses to pertussis in the absence of LCC. Importantly, resting levels of CD25 expression did not differ significantly between $HCMV^+$ and $HCMV^-$ donors (Fig. 2A), and there was no difference in the potential of T cells from $HCMV^-$ and $HCMV^+$ donors to produce IL-2 in response to pertussis Ag (Supplemental Fig. 1B and 1C). Furthermore, there is no intrinsic difference in the ability of NK cells from $HCMV^+$ and $HCMV^-$ donors to degranulate in response to CD16 cross-linking or K562 stimulation (Supplemental Fig. 1D and 1E). However, NK cell $CD25^+$, $CD25^+IFN\text{-}\gamma^+$, and $CD107a^+$ expression in response to HCC (high concentrations of IL-12 and IL-18) were all significantly higher in $HCMV^-$ compared with $HCMV^+$ donors (Fig. 2A–C). Analysis of

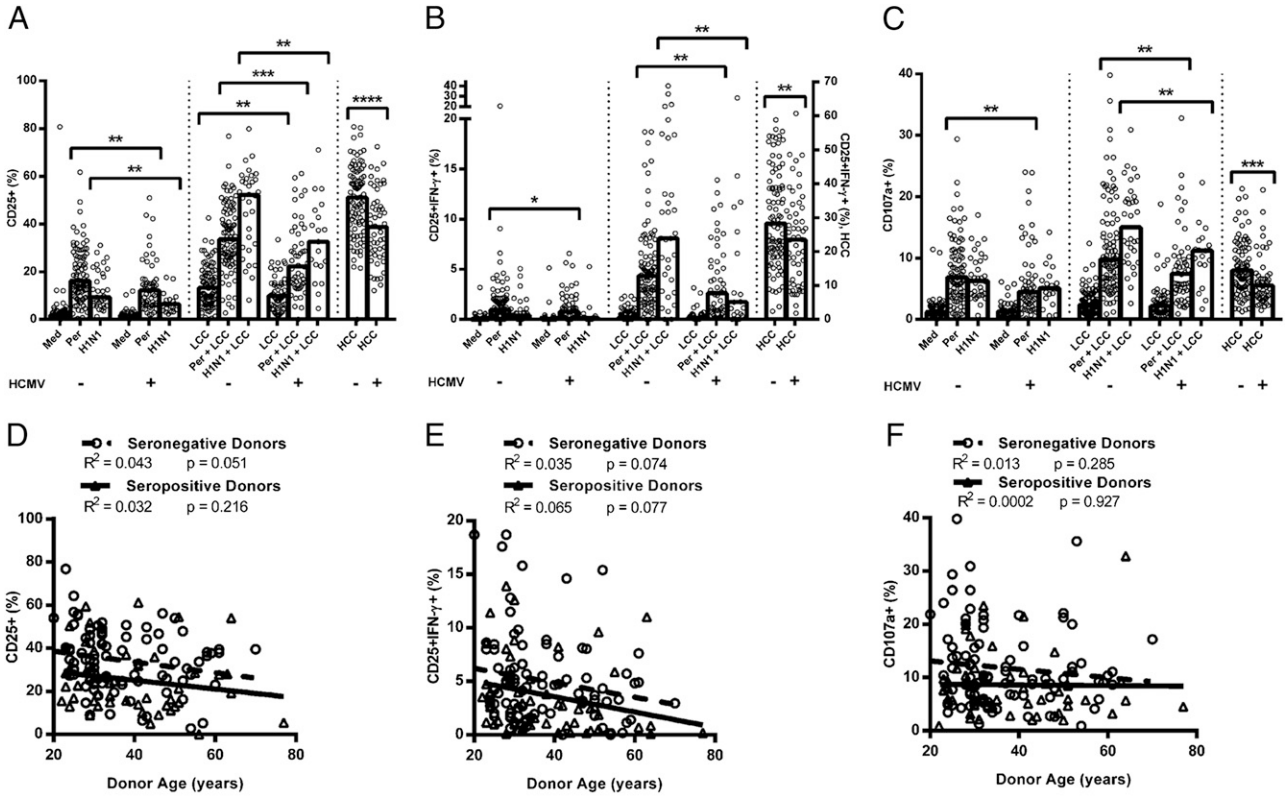

FIGURE 2. NK cell responses to vaccine Ag are affected by HCMV infection. PBMCs were cultured in vitro for 18 h with medium alone, LCC, killed whole-cell pertussis (Per), inactivated whole H1N1 influenza virus (H1N1), Per + LCC, H1N1 + LCC, or HCC. Donors were stratified into $HCMV^-$ (−) and $HCMV^+$ (+) groups. Responses were measured as the percentage of NK cells expressing CD25 (**A**), coexpressing CD25/IFN-γ (**B**) or CD107a (**C**). Bivariate regression of age against responses to Per + LCC was performed for the percentage of NK cells expressing CD25 (**D**), CD25/IFN-γ (**E**), and CD107a (**F**). Each data point represents one donor, $n = 152$, except for H1N1 and H1N1 + LCC where $n = 52$. Bar graphs denote medians. NB, all Ag stimulations induced statistically significant increases in expression of CD25, CD25/IFN-γ, and CD107a over background (medium alone for pertussis/H1N1, or LCC for pertussis+LCC/H1N1+LCC; $p < 0.05$ in all cases), except that H1N1 did not induce a significant increase in $CD25^+IFN\text{-}\gamma^+$ NK cells in $HCMV^+$ donors ($p = 0.416$). Data were analyzed in Prism using, one-tailed Mann–Whitney U tests. $^{****}p \leq 0.0001$, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

this response by $CD56^{bright}$ and $CD56^{dim}$ subsets reveals that the effect of HCMV status is due entirely to an effect within the $CD56^{dim}$ subset (Supplemental Fig. 2D–F).

In addition to consistently lower NK cell responses to vaccine Ags in $HCMV^+$ individuals, there was a trend for CD25 and CD25/IFN-γ responses to pertussis (with or without LCC) to decline with increasing age (Fig. 2D and 2E). This was statistically significant for the cohort as a whole ($CD25^+$ pertussis: $R^2 = 0.0549$, $p = 0.0052$; $CD25^+$ pertussis + LCC: $R^2 = 0.0453$, $p = 0.0122$; $CD25^+IFN\text{-}\gamma^+$ pertussis: $R^2 = 0.0379$, $p = 0.0203$; $CD25^+IFN\text{-}\gamma^+$ pertussis + LCC: $R^2 = 0.0478$, $p = 0.0095$), but not when analyzed separately for $HCMV^-$ and $HCMV^+$ donors due to decreased power. There was no effect of age on CD107a expression (pertussis: $R^2 = 0.00491$, $p = 0.4089$; pertussis + LCC: $R^2 = 0.00879$, $p = 0.272$; Fig. 2F), which is consistent with maturation of the NK cell repertoire, and therefore decreased sensitivity to exogenous cytokines, but maintained cytotoxicity, during normal aging (reviewed in Ref. 37) and increasing NK cell differentiation (8, 9). Importantly, the effect of HCMV infection on impaired NK cell responses to pertussis and H1N1 is entirely independent of the association between age and NK cell function. In line with this conclusion, adjusting for age by parametric regression did not alter the conclusions of the study (Table II).

Overall, NK cell responses did not differ significantly between males and females, although there was a trend for median responses to be higher in women than in men, and this reached statistical significance ($p < 0.05$) for the IFN-γ response to pertussis + LCC in $HCMV^+$ donors (data not shown). Because the proportion of female subjects differed between the $HCMV^-$ and $HCMV^+$ groups (Table I), the data in Fig. 2 were reanalyzed, adjusting for sex, as well as age, using parametric regression (Table II). After adjustment, CD25/IFN-γ and CD107a expression in response to vaccine alone (i.e., without LCC) are no longer significantly different between $HCMV^-$ and $HCMV^+$ donors, but responses to vaccine with LCC, and responses to HCC, remain significantly lower in $HCMV^+$ compared with $HCMV^-$ donors.

Finally, no associations were observed between anti-HCMV titer and any NK cell responses among the $HCMV^+$ subjects, and there was no effect of *NKG2C* genotype (which may affect NK cell differentiation) (30, 38, 39) on NK cell responses (data not shown).

NK cell differentiation only partially explains reduced responses to vaccines in $HCMV^+$ donors

We hypothesized that reduced cytokine-mediated NK cell responses among $HCMV^+$ donors would reflect expansion of the highly differentiated $CD56^{dim}CD57^+NKG2C^+$ NK cell subset, which is known to be hyporesponsive to cytokines (12). Indeed, ex vivo analysis confirmed observations from previous studies that $HCMV^+$ donors had lower proportions of $CD56^{dim}CD57^-$ NK cells and higher proportions of $CD56^{dim}CD57^+$ NK cells than did $HCMV^-$ donors (Fig. 3A and 3B); there was no difference between the groups in the proportion of cells with intermediate CD57 expression ($CD56^{dim}CD57^{int}$, gating shown in Fig. 3A). Consistent with previous work (10–12, 16, 17), HCMV seropositivity was also associated with a higher proportion of $CD16^+$ (Fig. 3C) and $NKG2C^+$ (Fig. 3D) cells, and a lower proportion of $NKG2A^+$ cells (Fig. 3E), within the total NK cell population. Moreover, HCMV seropositivity was correlated with a lower proportion of $CD57^-NKG2C^-$ cells and a higher proportion of $CD57^+NKG2C^+$ cells within the $CD56^{dim}$ NK cell population (Fig. 3F).

Although the increased proportion of $CD56^{dim}CD57^+$ NK cells among $HCMV^+$ donors likely contributes to their reduced responsiveness to cytokines, we also observed significantly reduced CD25, CD25/IFN-γ, and CD107a expression in response to both pertussis and H1N1 *within* individual NK cell subsets. This was especially evident among $CD56^{dim}CD57^+$ cells and for cultures containing LCC (Fig. 4A–F), but this was also the case for cultures stimulated with vaccine alone (Supplemental Fig. 3G–I and 3M–O).

Similarly, when cells were grouped by expression of CD57 and NKG2C, we found that responses to pertussis with LCC were lower among $NKG2C^+$ NK cells than among $NKG2C^-$ cells (Fig. 4G–I). This association was statistically significant for $CD57^+$ NK cells of $HCMV^+$ donors, but evaluation of the $HCMV^-$ cohort lacked statistical power as too few donors had sufficient $NKG2C^+$ cells to allow a robust analysis. Interestingly, however, responses of all

Table II. NK cell responses to vaccine Ags by HCMV status after adjusting for sex and age

		Adjusted for Sex and Age	
Stimulus	Parameter (Total NK Cells)	Effect (95% CI)[a]	p^b
Pertussis	$CD25^+$	−4.4 (−8.3, −0.5)	<u>0.014</u>
	$CD25^+IFN\text{-}\gamma^+$	−0.5 (−1.2, 0.3)	0.125
	$CD107a^+$	−1.5 (−3.4, 0.5)	0.071
Pertussis + LCC	$CD25^+$	−8.5 (−13.7, −3.4)	<u>0.001</u>
	$CD25^+IFN\text{-}\gamma^+$	−1.5 (−2.8, −0.1)	<u>0.020</u>
	$CD107a^+$	−2.9 (−5.5, −0.3)	<u>0.016</u>
H1N1	$CD25^+$	−5.4 (−9.5, −1.3)	<u>0.005</u>
	$CD25^+IFN\text{-}\gamma^+$	−0.4 (−1.1, 0.4)	0.158
	$CD107a^+$	−1.8 (−3.9, 0.3)	<u>0.049</u>
H1N1 + LCC	$CD25^+$	−12.2 (−22.6, −1.8)	<u>0.011</u>
	$CD25^+IFN\text{-}\gamma^+$	−5.1 (−10.4, 0.1)	<u>0.027</u>
	$CD107a^+$	−5.1 (−8.9, −1.5)	<u>0.004</u>
HCC	$CD25^+$	−11.3 (−16.7, −6.0)	<u><0.0001</u>
	$CD25^+IFN\text{-}\gamma^+$	−6.5 (−11.4, −1.7)	<u>0.005</u>
	$CD107a^+$	−2.1 (−3.5, −0.6)	<u>0.004</u>

A regression analysis was performed in STATA to adjust for sex and age when comparing NK cell responses to pertussis ($^{-/+}$ LCC), H1N1 ($^{-/+}$ LCC), and HCC between $HCMV^-$ and $HCMV^+$ donors. The response was quantified by the percentage of total NK cells expressing CD25, CD25/IFN-γ ($CD25^+IFN\text{-}\gamma^+$), and CD107a.

[a] Effect (coefficient), with 95% confidence interval (CI), represents the change in the mean percentage of NK cells responding in $HCMV^+$ donors as compared with $HCMV^-$ donors.

[b] The p value refers to the significance of the difference in response between $HCMV^-$ and $HCMV^+$ donors after adjusting for sex and age. The p values < 0.05 are underlined.

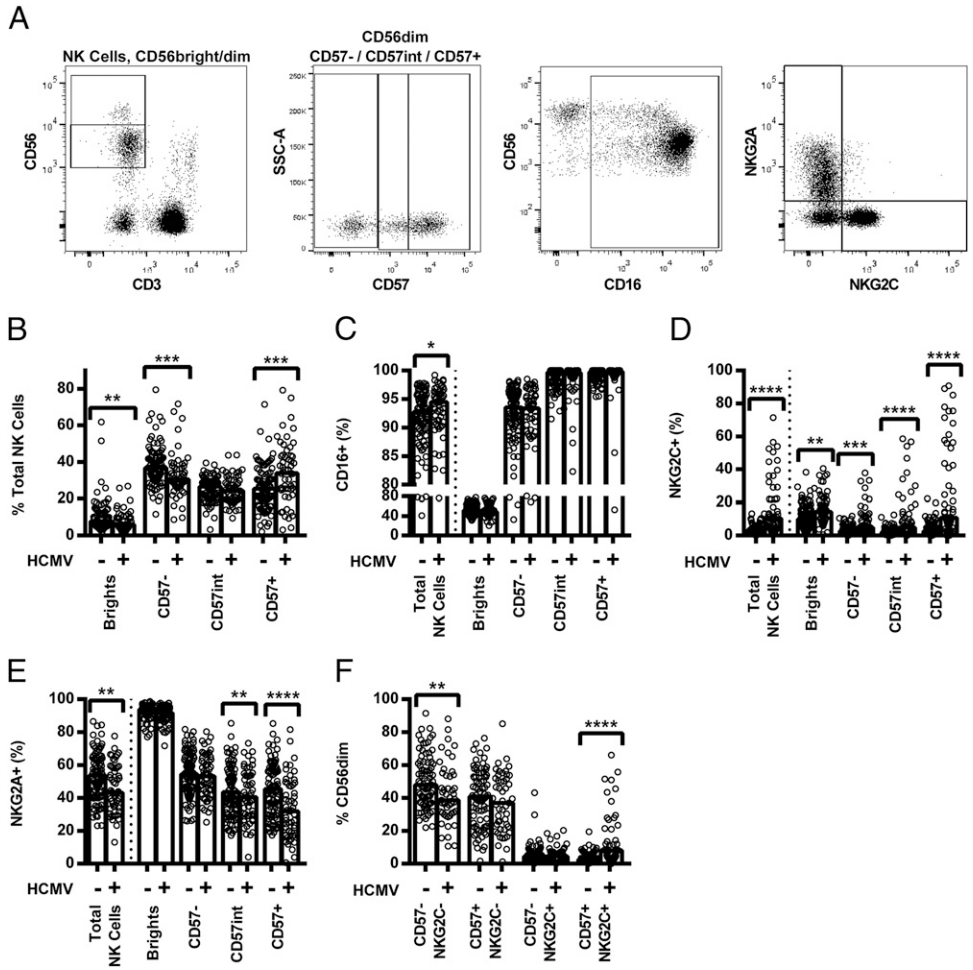

FIGURE 3. Comparison of ex vivo expression of NK cell markers and receptors in HCMV$^-$ and HCMV$^+$ donors. PBMCs were analyzed ex vivo for surface expression of CD56, CD57, CD16, NKG2C, and NKG2A, as shown by representative flow cytometry plots (**A**). Proportions of total NK cells in the CD56^{bright}, CD56^{dim}CD57$^-$, CD56^{dim}CD57^{int}, and CD56^{dim}CD57$^+$ subsets were compared between HCMV$^-$ and HCMV$^+$ donors (**B**), as was expression of CD16 (**C**), NKG2C (**D**), NKG2A (**E**), and CD57/NKG2C (**F**, CD56^{dim} only). The percentages of cells expressing each marker in HCMV$^-$ (−) and HCMV$^+$ (+) donors were compared using two-tailed Mann–Whitney *U* tests. Each data point represents one donor, $n = 152$; bar graphs denote medians. $^{****}p \leq 0.0001$, $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

four subsets were significantly lower among HCMV$^+$ donors than among HCMV$^-$ donors (Fig. 4G–I), despite minimal differences in responses to LCC alone (Supplemental Fig. 3A–F). These data indicate that the reduced response of HCMV$^+$ donors reflects differences in the intrinsic responsiveness of NK cells within a subset, as well as differences in the distribution of these subsets. Although the level of expression (median fluorescence intensity [MFI]) of both CD57 and NKG2C was higher on CD56^{dim}CD57$^+$ NK cells in HCMV$^+$ donors compared with HCMV$^-$ donors (median MFI CD57: 13,526 versus 10,575, $p = 0.0032$; median MFI NKG2C: 141 versus 80.9, $p < 0.0001$, data not shown), there was no significant association between CD57 and NKG2C expression levels and NK cell responsiveness in HCMV$^+$ donors (data not shown).

Because only some HCMV$^+$ individuals have obvious expansion of the CD56^{dim}CD57$^+$NKG2C$^+$ subset, we considered whether NK responses might differ between HCMV$^+$ individuals with and without this expanded population. Sixteen of 55 (29%) HCMV$^+$ donors demonstrated expansion of the CD56^{dim}CD57$^+$NKG2C$^+$ subset (defined as % CD56^{dim}CD57$^+$NKG2C$^+$ cells greater than the mean + 3 SD of that in HCMV$^-$ donors), and NK cells from these donors tended to respond less robustly than did cells from HCMV$^+$ donors without this expansion (Fig. 5). Importantly, there was evidence by trend analysis for decreasing NK cell responsiveness with HCMV infection and then with HCMV infection plus expansion of the CD56^{dim}CD57$^+$NKG2C$^+$ subset (Fig. 5). This confirms that although expansion of the CD56^{dim}CD57$^+$ NKG2C$^+$ subset is associated with loss of NK cell responsiveness in vaccine recall assays, cells of HCMV$^+$ donors respond less well than do cells of HCMV$^-$ donors, irrespective of NKG2C expression.

HCMV infection is associated with altered expression of cytokine receptors by NK cells

Although there was a clear role for specific IgG in induction of CD25, CD25/IFN-γ, and CD107a expression (Fig. 1), impairment of CD16-mediated signaling seemed an unlikely explanation for reduced NK cell responsiveness because HCMV$^+$ individuals have a higher frequency of CD16$^+$ NK cells (Fig. 3C), cells from HCMV$^+$ and HCMV$^-$ donors responded equally well to CD16 cross-linking (Supplemental Fig. 1D), and use of pooled AB plasma for in vitro assays ensured that specific IgG concentrations were consistent in all assays.

In contrast, differences between HCMV$^+$ and HCMV$^-$ donors were most marked in cultures containing LCC (Fig. 2) and in cultures with high concentrations of the cytokines IL-12 and IL-18 (HCC; Fig. 6A–C), suggesting that differences in expression of cytokine receptors might explain our observations. Although there

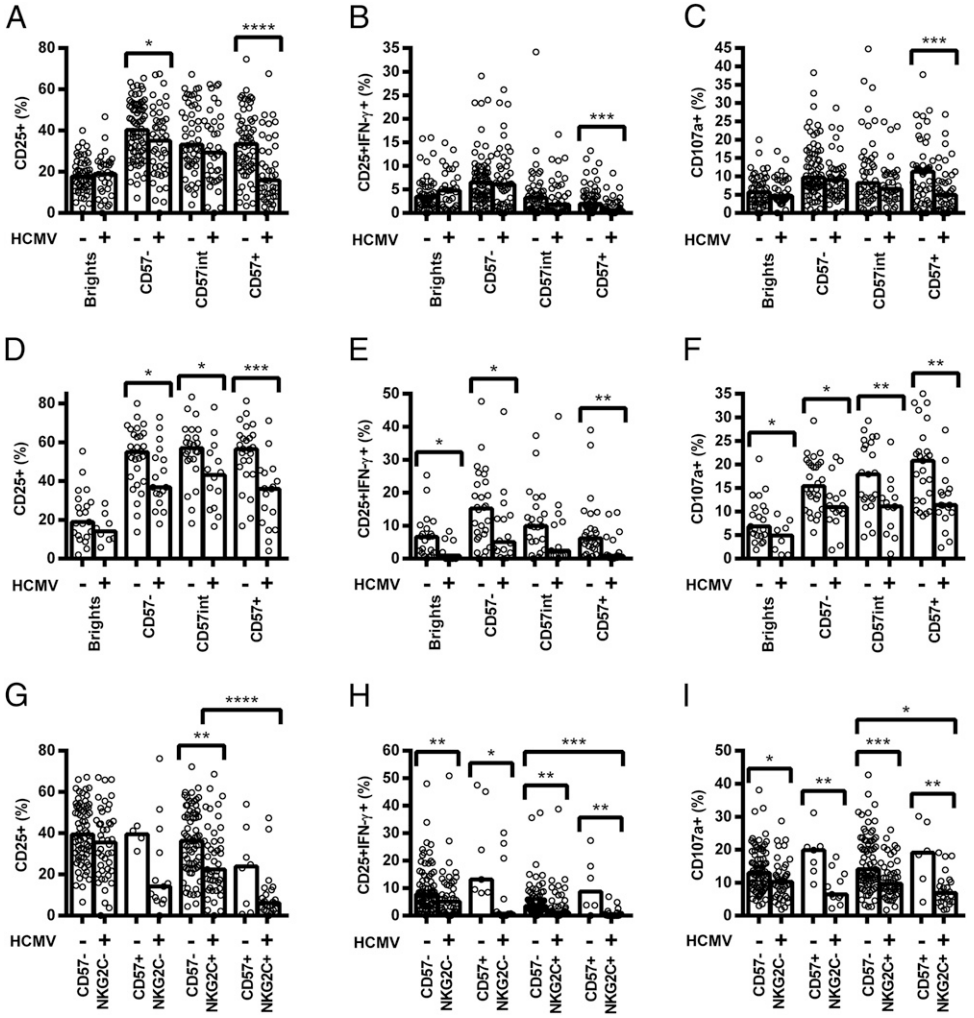

FIGURE 4. HCMV infection affects vaccine Ag responses of all NK cells, irrespective of their differentiation status. PBMCs were cultured in vitro for 18 h with killed whole-cell pertussis with LCC (pertussis + LCC) (**A**–**C** and **G**–**I**) or inactivated whole H1N1 influenza virus with LCC (H1N1 + LCC) (**D**–**F**). Responses were measured as the percentage of cells expressing CD25 (A, D, and G), CD25/IFN-γ (B, E, and H), and CD107a (C, F, and I) by CD56/CD57-defined subsets (A–F) or $CD56^{dim}$ CD57/NKG2C-defined subsets (G–I) and compared between $HCMV^-$ (−) and $HCMV^+$ donors (+). Data were analyzed using one-tailed Mann–Whitney *U* tests. Each data point represents one donor, $n = 152$ (A–C and G–I) or $n = 52$ (D–F); bar graphs denote medians. NB, for CD57/NKG2C-defined subsets, $CD57^{int}$ cells were grouped together with $CD57^-$ cells. $****p \leq 0.0001$, $***p < 0.001$, $**p < 0.01$, $*p < 0.05$.

was no difference in resting (ex vivo) expression of IL-12Rβ2 on any NK cell subset (Fig. 6D and 6E), IL-12Rβ2 was significantly upregulated on the total NK cell population in $HCMV^-$ but not from $HCMV^+$ donors after culture with HCC (Fig. 6F). Moreover, and consistent with data showing associations between acquisition of CD57 and decreased IL-18Rα expression (6, 8, 9), resting NK cells from $HCMV^+$ donors were significantly less likely than cells from $HCMV^-$ donors to express IL-18Rα, and this difference was especially marked in the (expanded) $CD56^{dim}CD57^+$ NK cell subset (Fig. 6G and 6H).

Discussion

During secondary immune responses, both $CD4^+$ T cell–derived IL-2 and Ag–Ab immune complexes induce "Ag-specific" NK cell activation, allowing NK cells to act as effectors of the adaptive immune response and to contribute to postvaccination immunity by secretion of IFN-γ and/or by cytotoxicity (3–6, 14). In this study, we demonstrate for the first time, to our knowledge, that the contribution of NK cells to adaptive immune responses is affected by HCMV infection: NK cells from $HCMV^+$ donors respond significantly less well than cells from $HCMV^-$ donors to killed whole-cell pertussis or inactivated whole H1N1 influenza virus. The effect of HCMV infection on NK cell responsiveness is independent of age, sex, or anti-HCMV IgG titer.

Our data also demonstrate for the first time, to our knowledge, that there is an additive effect between the cytokine and the IgG pathways driving NK cell IFN-γ production, because both IgG depletion and IL-2 blockade reduced NK cell IFN-γ responses in response to stimulation of PBMCs with pertussis vaccine. Of particular interest, IgG depletion markedly reduced Ag-induced CD25 expression on NK cells. We propose that CD16 cross-linking by immune complexes upregulates CD25 expression, increasing sensitivity to T cell–derived IL-2, and thereby enhancing IFN-γ production. However, CD16 cross-linking is not essential for upregulation of CD25, because this can be induced by Ag alone, presumably in response to IL-12 and IL-18 produced by APCs (6, 40–42). Release of cytotoxic granules, as measured by upregulation of CD107a on the cell surface, is also inhibited by IgG depletion but is unaffected by IL-2 blockade, suggesting that NK cells could act as effectors of the adaptive response through ADCC in the absence of memory T cells, providing there is sufficient circulating Ab.

However, whereas IgG depletion also decreased H1N1-induced CD25 expression on NK cells, H1N1 induction of IFN-γ was

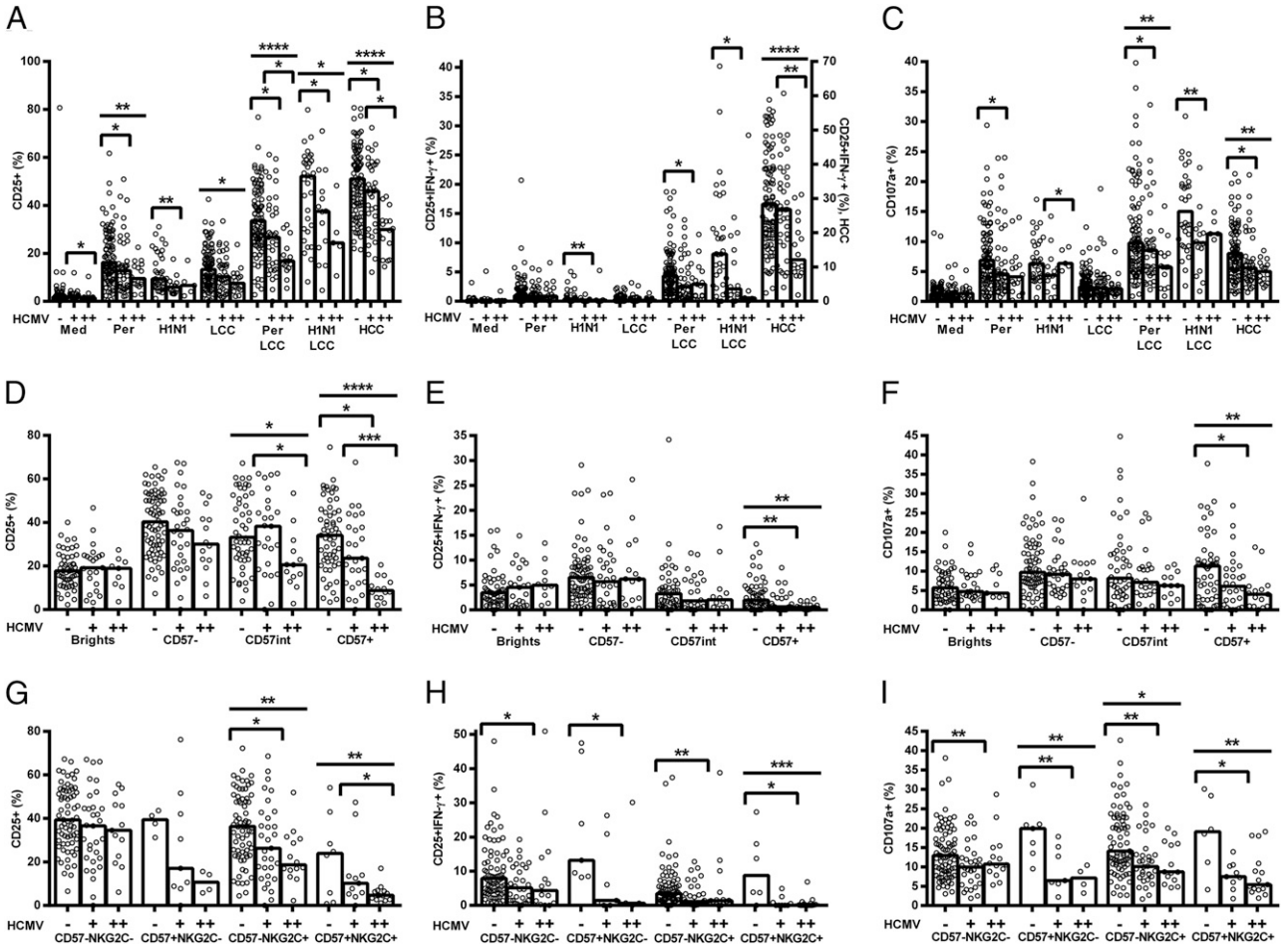

FIGURE 5. NK cell responses of HCMV$^+$ donors with or without the characteristic CD56^{dim}CD57$^+$NKG2C$^+$ expansion. PBMCs were cultured in vitro for 18 h with medium alone, LCC, killed whole-cell pertussis (Per), inactivated whole H1N1 influenza virus (H1N1), Per + LCC, H1N1 + LCC, or HCC. Donors were stratified into HCMV$^-$ (−), HCMV$^+$ without expansion of CD56^{dim}CD57$^+$NKG2C$^+$ cells (+), and HCMV$^+$ with expansion of CD56^{dim}CD57$^+$ NKG2C$^+$ cells (++). Responses are expressed as the percentage of total NK cells expressing CD25 (**A**), coexpressing CD25/IFN-γ (**B**), or expressing CD107a (**C**). CD57-defined (**D**–**F**) or CD57/NKG2C-defined subsets (**G**–**I**) were analyzed for responses to pertussis with LCC for CD25 (D and G), CD25/IFN-γ (E and H), and CD107a (F and I). Data were analyzed in Prism using one-tailed Mann–Whitney *U* tests to compare responses between HCMV$^+$ donors and either HCMV$^-$ donors or HCMV^{++} donors. ANOVA for linear trend (from − to + to ++) was also performed for each functional readout. Each data point represents one donor, $n = 152$, except for H1N1 and H1N1 + LCC where $n = 52$. Bar graphs denote medians. ****$p \leq 0.0001$, ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

significantly enhanced in the absence of IgG. We have observed that individual NK cells tend to either produce IFN-γ or degranulate (but not both; unpublished data), suggesting that inhibiting the degranulation response to H1N1 by removing IgG skews the response toward IFN-γ production. However, given the limited effect of IgG depletion on H1N1-induced degranulation, it is unclear why this should be the case. Indeed, expression of CD107a in response to H1N1 seems to be relatively unaffected by either IL-2 blockade or IgG depletion. This suggests that H1N1-driven degranulation may be affected by other stimuli, such as type I IFNs (43, 44).

We had hypothesized that decreased responses to vaccines in HCMV$^+$ donors would be attributable to a redistribution of the NK cell repertoire. HCMV infection drives the expansion of a CD56^{dim}CD57$^+$NKG2C$^+$ subset of NK cells (11, 16, 17, 45), which display a highly differentiated phenotype, including reduced responsiveness to exogenous cytokine stimulation (8, 9) and epigenetic changes at the *IFNG* locus (46). These phenotypic and functional changes are similar to those observed during aging (15, 47), and comparisons have been drawn between the effects of HCMV and immunosenescence (48). Because our previous work has indicated that NK cell IFN-γ production after restimulation with vaccine Ags is cytokine dependent (3), we predicted that fewer NK cells from HCMV$^+$ donors would produce IFN-γ in response to pertussis or influenza Ags because of the reduced capacity of the expanded CD56^{dim}CD57$^+$NKG2C$^+$ subset to respond to cytokines. Ex vivo analyses confirmed that HCMV$^+$ donors had higher proportions of CD56^{dim}CD57$^+$ and CD56^{dim} CD57$^+$NKG2C$^+$ NK cells than did HCMV$^-$ donors, and functional analysis confirmed that few of the highly differentiated CD57$^+$ NK cells produced IFN-γ after Ag stimulation. Interestingly, however, our data also show that, irrespective of their CD57/NKG2C surface phenotype, NK cells from HCMV$^+$ donors are less likely to produce IFN-γ in response to vaccines than are cells from HCMV$^-$ donors. In other words, there are pronounced functional differences between HCMV$^+$ and HCMV$^-$ donors *within* NK cell subsets. The reduced NK cell IFN-γ response to vaccine Ags in HCMV$^+$ donors is therefore not simply due to expansion of the CD56^{dim}CD57$^+$NKG2C$^+$ subset. Although acquisition of NKG2C was functionally relevant (associated with reduced IFN-γ and degranulation responses), it was not sufficient to explain the reduced responsiveness of cells from HCMV$^+$ donors.

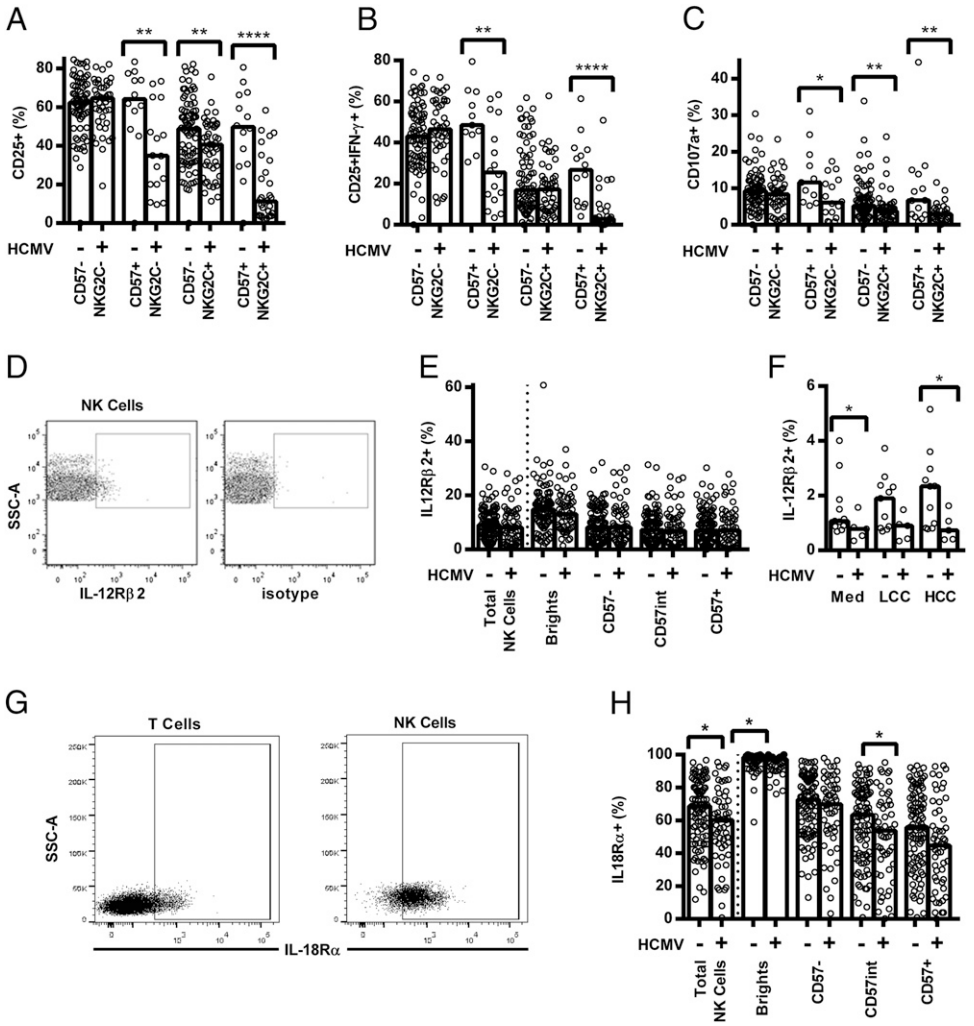

FIGURE 6. Decreased cytokine responsiveness and decreased cytokine receptor expression by NK cells from HCMV$^+$ donors. (**A**–**C**) PBMCs were cultured in vitro for 18 h with an HCC. Responses were measured as the percentage of CD56^{dim} CD57/NKG2C-defined cells expressing CD25 (A), CD25/IFN-γ (B), and CD107a (C), and compared between HCMV$^-$ (−) and HCMV$^+$ donors (+). (**D**–**F**) NK cells were analyzed for surface expression of IL-12Rβ2 using an mIgG1 PECy5-conjugated isotype control to set the gate (D). Total NK cells (E and F) and CD56/CD57-defined subsets (E) were analyzed ex vivo (E) and after 18 h culture in vitro with LCC or HCC (F). (**G** and **H**) NK cells were also analyzed for IL-18Rα surface expression using the T cell population to set the IL-18Rα gate (G), for total NK cells and CD56/CD57-defined subsets ex vivo (H). HCMV$^-$ and HCMV$^+$ donors were compared using one-tailed (A–C) or two-tailed (E, F, and H) Mann–Whitney *U* tests. Each point represents one donor, $n = 152$ (A–C, E, and H) or $n = 16$ (F); bar graphs denote medians. $^{****}p \leq 0.0001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

Although further studies are required to define the "within subset" effects of HCMV infection, our data suggest that reduced expression of IL-18Rα or reduced ability to upregulate IL-12Rβ2 among NK cells from HCMV-infected individuals may partially explain their failure to produce IFN-γ. Although decreasing expression of IL-12Rβ2 and IL-18Rα has been associated with CD57 expression, this is the first demonstration, to our knowledge, that there are differences in cytokine receptor expression between HCMV$^+$ and HCMV$^-$ donors and it is possible to see how each of these might affect NK cell responses. Higher resting levels of IL-18Rα expression would increase the sensitivity of NK cells to low concentrations of IL-18 being produced by APCs in response to innate receptor ligands in whole-cell pertussis or inactivated influenza virus. IL-18 signaling upregulates CD25 (49), thereby increasing sensitivity to IL-2. IL-2 signaling might then upregulate IL-12R2β (50, 51), allowing IL-12 to synergize with IL-2 to drive IFN-γ production (3, 40, 52), while also generating a positive feedback loop in which IL-12 signaling upregulates IL-18Rα (53, 54), IL-18 signaling, and CD25. However, although cytokine receptor expression is likely to play a role in determining NK cell responsiveness to vaccine Ags in HCMV$^-$ and HCMV$^+$ donors, the biological relevance of small changes in surface expression on IL-12Rβ2 needs to be demonstrated. Moreover, although we have no evidence to suggest that T cell IL-2 production in response to vaccine Ags is affected by HCMV infection, future studies will need to determine the extent to which concomitant changes in APC function during HCMV infection also affect NK cell responses.

We had initially considered NK cell degranulation during vaccine restimulation to be a result of CD16 cross-linking by IgG immune complexes, as suggested by the IgG depletion data and accepted models of ADCC. The expectation was, therefore, that although IFN-γ responses might be impaired, NK cell degranulation responses would be sustained in HCMV$^+$ donors. Indeed, cross-linking with anti-CD16 Ab induced equivalent levels of CD107a upregulation. It was, therefore, somewhat surprising that degranulation responses to vaccine were lower in HCMV$^+$ donors than in HCMV$^-$ donors. However, degranulation responses to HCC were also lower in HCMV$^+$ donors, supporting the notion of synergy between the cytokine and CD16 pathways, and adding

weight to the suggestion that HCMV infection may affect cytokine receptor expression.

Our findings have potentially important implications. HCMV infection is a known risk factor for all-cause mortality in adults (55), and perinatal HCMV infection is associated with slower growth and increased rates of hospitalization in African children (56). The underlying biology of these relationships is unknown, but reduced responsiveness to vaccination or reduced resilience in the face of infection are plausible explanations. Distorted T cell and NK cell phenotypes in $HCMV^+$ individuals have been widely reported (15, 57–59), giving credence to the possibility that adaptive immune responses may be less effective in infected individuals. Further work will need to address the clinical consequences of altered NK cell responses to infection and vaccination in HCMV-infected individuals.

To our knowledge, this is the first published study of the effect of HCMV infection on NK cell responses to vaccine Ags. When compared with the marked effect of HCMV on cellular immune responses in our adult cohort, the modest phenotype seen in the infant studies (24, 25) raises the intriguing question whether the duration of HCMV infection affects vaccine responses. We have previously shown in an African population that, with near-universal infant HCMV infection, the characteristic "adult HCMV" NK cell profile is reached by early adolescence (30). The majority of our donors are of European or North American origin (data not shown), suggesting that they may have been infected in adolescence or adulthood (60, 61), potentially explaining some of the heterogeneity in the responses we see within the $HCMV^+$ group. Similarly, there will be variation among our donors in time since vaccination (pertussis) or infection (H1N1), and it is likely that relatively low IFN-γ responses we observe in comparison with earlier studies (3) is due to the much longer interval between primary and secondary exposures to Ag. Future studies will need to assess whether the duration of HCMV infection is a risk factor for altered NK responses and whether this manifests itself as reduced responsiveness to active vaccination and reduced vaccine efficacy.

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Disclosures

The authors have no financial conflicts of interest.

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www.lshtm.ac.uk

Observational / Interventions Research Ethics Committee

Eleanor Riley
Professor of Immunology
IID/ITD
LSHTM

14 January 2013

Dear Professor Riley,

Study Title: **The role of human cytomegalovirus (HCMV) in driving phenotypic and functional differentiation of Natural Killer cells**
LSHTM ethics ref: **6324**

Thank you for your letter of 11 January 2013, responding to the Observational Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

Approval is dependent on local ethical approval having been received, where relevant.

Approved documents

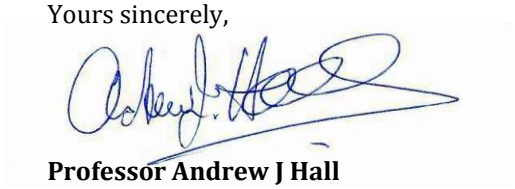
The final list of documents reviewed and approved by the Committee is as follows:

Document	Version	Date
LSHTM ethics application	n/a	
Protocol	V2	11/01/2013
Information Sheet NK CMV	V2.A1	13/11/2012
Consent form	V1	09/10/2012
HCMV study-Data capture form	V1	26/10/2012
Sample recruitment email		

After ethical review

Any subsequent changes to the application must be submitted to the Committee via an E2 amendment form. All studies are also required to notify the ethics committee of any serious adverse events which occur during the project via form E4. At the end of the study, please notify the committee via form E5.

Yours sincerely,



Professor Andrew J Hall
Chair
ethics@lshtm.ac.uk
http://intra.lshtm.ac.uk/management/committees/ethics/

Observational/Interventions Research Ethics Committee
Application to conduct a study involving human participants

Please ensure you download and complete the latest version of this form from the intranet:
http://intra.lshtm.ac.uk/management/committees/ethics/

This form should be completed and emailed along with all relevant attachments to ethics@lshtm.ac.uk. Attachments should be appended as a single file.

For use of Ethics Committee only	Application No.	Date received
	Response deadline	Date approval notification

Title of Project The role of human cytomegalovirus (*HCMV*) in driving phenotypic and functional differentiation of Natural Killer cells.	
Name of Chief Investigator (CI) (and institution if not LSHTM)	Eleanor Riley
Appointment Held (or Research student) Department/ Faculty	Professor of Immunology
LSHTM lead investigator *(if different from above)*	IID/ITD
LSHTM contact for correspondence *(if different from above)*	
Other personnel involved	Dr Matthew White (IID, researcher) Dr Martin Goodier (IID) Carolynne Stanley (IMMU research co-ordinator and ITD technician) Miss Carolyn Nielsen (IID, PhD student)
If Research student: Name, electronic signature and approval of Supervisor (or attach email approval)	Not applicable

Definition of interventions study for LSHTM review purposes: Interventional studies include all trials based on random allocation of interventions and also non-randomised interventions where participants or groups of participants are given treatments (of whatever nature) that they would not otherwise be receiving in the ordinary course of events and which are allocated by the investigators.		
Is this an intervention study?	YES	
	NO	X

Fastrack: Applications in the following categories will be dealt with by Chair's Action)		
Is this a DrPH Professional attachment?	YES	
	NO	X
Is this study using anonymised and unlinkable secondary datasets only	YES	
	NO	X
Has this study received NRES (NHS) approval? (*Approval to be attached)*	YES	
	NO	X
Is the CI of the main study based at another UK institution and has obtained ethics approval from their University Ethics Committee? *(Approval to be attached.)*	YES	
	NO	X

Checklist for submission

Please ensure you submit this application form electronically along with the relevant supporting documents listed below to ethics@lshtm.ac.uk . No hard copies are required.

Supporting documents should contain version numbers/dates where relevant, and all should be submitted within a single electronic file (separate to the application).

Please indicate supporting documents submitted with the application in the table below.

Document **(* provision is mandatory for all studies; **provision is mandatory for interventional studies)**	**Attached Y/N or n/a**	**Version number/date**
Application for ethical approval*	Y	
Protocol/amendments (including data collection forms, questionnaires)*	Y	
Informed consent forms/ updates and Information sheet/Written information to subjects *	Y	
Investigators' CVs*	Y	
Sponsor letter**	n/a	
Available safety information for interventional studies**	n/a	
Subject recruitment procedures (eg advertisements)	Y	
Investigator's brochure/Summary of product characteristics	n/a	
Information about payments/compensation to be provided to participants	n/a	
NRES (NHS)/other University ethical approval (if obtained)	n/a	
Local approval letter(s) (if obtained)		
Electronic signature or email approval of Supervisor (Research Degree students only)	n/a	
Written statement from company producing or providing any drug/appliance that it agrees to abide by the guidelines on compensation for non-negligent injury of the Association of the British Pharmaceutical Industry (ABPI)	n/a	

CIs/PIs must retain a copy of the approved version of this application and supporting documentation as part of their own research records in line with good practice. The Ethics Administrator will retain final copies on file for a period in accordance with School and other relevant record keeping requirements.

Please note: LSHTM risk assessment procedures are set out at http://intra.lshtm.ac.uk/safety/ (Travel Safety). All necessary procedures must be completed for all staff before fieldwork commences.

1.	Give an outline of the proposed project. Sufficient detail of the protocol must be given to allow the Committee to make an informed decision without reference to other documents. (Additional material should only be attached if considered absolutely necessary). *Max 300 words* **ATTACH FULL PROTOCOL TO THE ELECTRONIC SUBMISSION**
Answer: Expand box to fit	Objective: We wish to test the hypothesis that persistent infection with human cytomegalovirus (*HCMV*) drives phenotypic and functional differentiation of natural killer (NK cells) and may contribute to declining NK cell function and reduced responsiveness to vaccination. Summary of the study: 100 healthy adult volunteers, from among LSHTM staff and students, will be asked to provide 50ml of blood on a single occasion. Some donors may be asked to provide a subsequent sample in order to follow up any particularly interesting observations. Approximately 2ml of serum (in two 1ml aliquots) will be taken and frozen for subsequent anti-CMV antibody assays. Biological assays will be performed on enriched lymphocytes by flow cytometry to derive the phenotype of NK cells and their subsequent functional potential. This will also include measuring antigen-driven NK cell responses to known vaccine antigens such as tetanus toxoid, diphtheria toxin etc. HCMV status (seropositive or not) will be correlated with NK cell phenotype and function.
2.	State the intended value of the project. If this project or a similar one has been done before what is the value of repeating it? Give details of overviews and/or information on the Cochrane database. *Max 300 words* *This area is of increasing importance – please ensure you give a full response.*
	This is a novel piece of research. The potential for human CMV to affect NK cell phenotype is a known concept. However, to our knowledge, no one has performed functional assays to define the role of HCMV infection in functional differentiation of NK cells. Our preliminary data (from a study in The Gambia (Ref 1269)) indicate that a particular subset of NK cells (CD57hi, CD56Dim, NKG2A-, NKG2C+) accumulates with increasing age and that this NK subset is functionally impaired. We suspect that this population is enriched in Gambians due to HCMV infection. However, since HCMV seroprevalence in the Gambia is very high (only 4 of 467 donors were seronegative for HCMV), we cannot perform a robust comparison of HCMV infected and uninfected individuals in The Gambia. HCMV seroprevalence globally is around 40% and in adult Europeans is closer to 50% allowing us to assess the role of this persistent infection in NK cell responses in UK resident donors. HCMV is a member of the herpesviridae family and is generally passed vertically (during pregnancy or in the perinatal period) or horizontally through child-child contact. Infection goes un-noticed in most healthy individuals but can cause serious illness in immune-compromised patients (HIV positive, transplant recipients etc). HCMV infection is also known to affect long term health outcomes, but the mechanism is unknown. NK cells are however known to be the primary cell type responsible for killing CMV infected target cells. Moreover, NK cells are being increasingly recognised as mediators of vaccine induced immunity to a range of diseases (Rabies, TB and Tetanus). If NK cell function deteriorates with age in HCMV+ donors, this might explain declining vaccine efficacy in older individuals.
3.	Specify numbers, with scientific justification for sample size, age, gender, source and method of recruiting participants for the study. *Max 300 words*
	We will recruit up to a total of 100 individuals. All will be members of LSHTM or Birkbeck College (staff or students). Volunteers can be of any age (>18), equal numbers of male and female and should be in good health. Individuals on long term medication for any immune-related disorders will be excluded. Donors will be recruited by Carolynne Stanley (Immunology and Infection Department

	research co-ordinator) by email advertisement and posters/flyers on notice boards. Volunteers will also be recruited from among previously registered LSHTM donors.
4.	State the personal experience of the applicant and of senior collaborators in the study in the field concerned, and their contribution to the study.
	Professor Riley has more than 25 years research experience on immunity to malaria in humans. Dr Matthew White is an immunologist with experience in human and murine T cell and NK cell biology. Dr Goodier has more than 20 years research experience in human immunology and infectious disease. Carolyn Neilsen is a PhD student with more than 1 year laboratory experience in human immunology. Carolynne Stanley has managed the anonymous blood donor register at LSHTM for the last 10 years and is a qualified phlebotomist.
5.	State the likely duration of the project, and where it will be undertaken.
	Up to 3 years. Volunteers will be consented and bled at LSHTM (First Aid Room, Ground floor, Keppel St). All lab work and data analysis will be undertaken in the Immunology and Infection Department, LSHTM.
6.	Specify the procedures, including interviews, involving human participants with brief details of actual methods. *Max 500 words*
	Informed Consent. Brief questionnaire (age, sex, vaccination history). Venous blood sample (50ml)
7.	State the potential discomfort, distress or hazards that research participants may be exposed to (these may be physical, biological and/or psychological). What precautions are being taken to control and modify these? Include information on hazardous substances that will be used or produced, and the steps being taken to reduce risks.
	Potential discomfort, distress or hazards are limited to those associated with blood sampling. Pain associated with venesection is minimal; there is a risk of minor (and reversible) damage to the vein leading to bruising; a minority of subjects may experience transient dizziness, nausea or fainting. Blood donors at LSHTM are routinely asked to donate 50mls blood and we have not experienced any problems either medically or in terms of acceptability.
8. a)	Does the project involve pre-marketing use of a drug/appliance or a new use for a marketed product?
	NO
b)	Does the company producing or providing any drug/appliance (whether pre-marketed, new use for marketed product or licensed use of marketed product) agree to abide by the guidelines on compensation for non-negligent injury of the Association of the British Pharmaceutical Industry (ABPI)?

	If YES, a written statement from the company to this effect should be attached.
	NO
9.	Will payments be made to participants? These should usually not be for more than travelling expenses and/or loss of earnings and must not represent an inducement to take part. If YES give details and justification. Please supply copies of information about payments/compensation that will be provided to participants
	NO
10.	Specify how confidentiality will be maintained with respect to the data collected. When small numbers are involved, indicate how possible identification of individuals will be avoided.
	A system of anonymous recruitment of blood donors for medical research has been running at LSHTM for over 10 years, co-ordinated by Ms Stanley. This system will be adopted for the proposed study. Identifying data on all volunteers will be entered into a secure database maintained by Carolynne Stanley and they will be given a unique study number which will be used to identify their questionnaires and samples. Only Ms Stanley will be aware of the identity of the individuals. Ms Stanley will co-ordinate recruitment and recall of volunteers.
11. a)	State the manner in which consent will be obtained. (**Note** the information sheet and consent form must be electronically appended and submitted with this application). Written consent is normally required. When this is not possible, a detailed explanation of the reasons should be given and a record of those agreeing kept. (see LSHTM SOP on Informed Consent for Research - LSHTM/SOP/014 http://intra.lshtm.ac.uk/trials/sops/sopsinpdf/sop_014_consent.pdf - although aimed at clinical trials the principles apply to all studies) If research is on human tissue samples, investigators must refer to guidance notes at http://intra.lshtm.ac.uk/support/research/humantissueact.html If any photographs are to be taken, whether for teaching or research purposes, ensure that the participant's consent to their use has been given in line with the provisions in *British Medical Journal, 1998, **316,** 1009-1011.*
	Written consent will be obtained, please see attached Participant Information Sheet and Consent form.
b)	Specify whether any subjects will be recruited from vulnerable groups? Please give details (This includes pregnant women, foetuses and neonates, children, prisoners, individuals with mental disability, individuals with learning difficulties, unconscious or severely ill, staff or students of LSHTM, other)
	No
c)	State the manner in which consent will be obtained from subjects recruited from vulnerable groups if this is not clear from 11a above (i.e. additional measures being put in place for these subjects?). (**Note** the information sheet and consent form must be electronically appended and submitted with this application)

	Not Applicable
12.	State what medical supervision is available and its location in relation to the participants.
	Ms Stanley is a trained first aider familiar with all the relevant procedures involved with phlebotomy at LSHTM.
13.	Will equivalent service or support to participants be available after the study ends? If NO, give details and describe steps to minimise loss of service or support.
	No. Participants will be expected to obtain medical services from their GP.
14. a)	For interventional trials (see definition on cover) Has, or will, the study be registered before the enrolment of the first participant on a publically accessible database? See http://www.who.int/ictrp/en/ for further information. *(A non-compulsory registry for observational studies in pharmacoepidemiology is available at http://www.encepp.eu/encepp_studies/index.html).*
	N/A
b)	Does the trial comply with Good Clinical Practice (GCP)? If no, explain why.
	N/A
c)	For clinical trials of medicines in the UK or EU please give details of CTA (Certificate of Clinical Trial Authorization).
	N/A.
d)	Is there a Data Safety Monitoring Board (DSMB) in place?
	N/A
15.	If the aim of the study is to improve treatment or management indicate how successful treatment would be continued or expanded.
	N/A
16.	Does this study involve the taking of blood samples and/or any other tissue?
	YES
17. a)	If YES Please list samples which will be taken.
	Peripheral blood (max 50mls) on a maximum of 2 occasions over a 3 month period
b)	Please confirm that you have undertaken the on-line training programme available at http://intra.lshtm.ac.uk/support/research/humantissueact.html and that you will ensure that any staff involved in the procedures for taking consent will also have undertaken an agreed training programme.
	YES
c)	If samples are taken overseas, will the samples be brought back to LSHTM at any time?
	N/A
18. a)	Where the research is to take place overseas, the Principal Investigator **must** seek ethical approval, through his/her overseas collaborators, in the country(s) concerned. Approval from the LSHTM Committee is dependent on local approval having been received. Please list the countries where research is being undertaken and arrangements being made to obtain local ethical and/or regulatory approval. Please electronically append copies of local approval letter(s) where this has already been obtained.

	N/A
b)	Where the research is taking place in the UK, please list other UK Committees from which approval is being sought.
	None
20.	Please give details of the funder and whether the funder sent the proposal out to Peer Review
	MRC UK.

Influenza Reagent
Influenza Antigen A/California/7/2009 (H1N1)v (NYMC-X179A) (Egg Derived)
NIBSC code: 09/146
Instructions for use
(Version 5.0, Dated 11/01/2013)

This material is not for in vitro diagnostic use.

1. INTENDED USE

Influenza antigen reagent 09/146 is prepared for single radial diffusion assay of A/California/7/2009 (H1N1)v antigens using an appropriate NIBSC antiserum reagent.

2. CAUTION

This preparation is not for administration to humans.

The material is not of human or bovine origin. As with all materials of biological origin, this preparation should be regarded as potentially hazardous to health. It should be used and discarded according to your own laboratory's safety procedures. Such safety procedures should include the wearing of protective gloves and avoiding the generation of aerosols. Care should be exercised in opening ampoules or vials, to avoid cuts.

3. UNITAGE

Antigen reagent 09/146 contains 50µgHA/ml.

4. CONTENTS

Country of origin of biological material: United Kingdom.
Antigen reagent 09/146 is prepared from formalin inactivated, partially purified A/California/7/2009 (H1N1)v (NYMC-X179A) virus which was suspended in PBSA buffer containing 1% (w/v) sucrose and processed for freeze drying as described in:
http://www.who.int/biologicals/reference_preparations/establishment/en/index.html

The reagent has been inactivated following validated procedures used to produce human influenza vaccine that is registered in the EU. This inactivated reagent has been shown to be free from residual infectious virus by testing according to the European Pharmacopeia Compendial Assay (monograph 0158).

5. STORAGE

-20°C
Please note: because of the inherent stability of lyophilized material, NIBSC may ship these materials at ambient temperature.

6. DIRECTIONS FOR OPENING

DIN ampoules have an 'easy-open' coloured stress point, where the narrow ampoule stem joins the wider ampoule body.
Tap the ampoule gently to collect the material at the bottom (labeled) end. Ensure that the disposable ampoule safety breaker provided is pushed down on the stem of the ampoule and against the shoulder of the ampoule body. Hold the body of the ampoule in one hand and the disposable ampoule breaker covering the ampoule stem between the thumb and first finger of the other hand. Apply a bending force to open the ampoule at the coloured stress point, primarily using the hand holding the plastic collar.
Care should be taken to avoid cuts and projectile glass fragments that might enter the eyes, for example, by the use of suitable gloves and an eye shield. Take care that no material is lost from the ampoule and no glass falls into the ampoule. Within the ampoule is dry nitrogen gas at slightly less than atmospheric pressure. A new disposable ampoule breaker is provided with each DIN ampoule.

7. USE OF MATERIAL

No attempt should be made to weigh out any portion of the freeze-dried material prior to reconstitution.
For all practical purposes each ampoule contains the same quantity of the substances listed above. Reconstitute the total contents of one ampoule of reagent with 1ml of distilled water. allow to stand for a minimum of 5 minutes before use to allow forcomplete solution of freeze dried material. antigen 09/146 should be used according to the method described by Wood, JM, Schild, GC, Newman RW and Seagrott, VA, journal of Biological Standardisation, 1977, 5, 237-247, with the following modification:

It is recommended that antigen reagent 09/146 and test A/California/7/2009 (H1N1)v virus antigens should be treated with Zwittergent 3-14 detergent (Calbiochem-Behring, La Jolla, CA, USA) before single single radial diffusion assay. Suitable incubation conditions are as follows:

450 microlitres of antigen are added to 50 microlitres of 10% (w/v) Zwittergent dteregent and incubated in covered containers for 30 minutes at room temperature (20-25°C). Dilutions of detergent treated antigens are then added to wells in single radial diffusion immunoplates and incubated at 20-25°C.

Reagent 09/146 should be used to assay A/California/7/2009 (H1N1)v antigens using an appropriate NIBSC antiserum reagent.

8. STABILITY

Reference materials are held at NIBSC within assured, temperature-controlled storage facilities. Reference Materials should be stored on receipt as indicated on the label.

NIBSC follows the policy of WHO with respect to its reference materials. Users of the material wishing to refer to the declared ampoule content for use in quantitative or semi-quantitative assay methods should note that the stated content of the material is based on a small collaborative study involving WHO Essential Regulatory Laboratories (ERLs) or Official Medicines Control Laboratories (OMCLs). Studies of recovery and stability of similar antigen preparations indicate that that recovery after ampouling is likely to be close to quantitative, and that no significant loss of content would be expected during storage over at least a 10 year period.

9. REFERENCES

N/A

10. ACKNOWLEDGEMENTS

N/A

11. FURTHER INFORMATION

Further information can be obtained as follows;
This material: enquiries@nibsc.org
WHO Biological Standards:
http://www.who.int/biologicals/en/
JCTLM Higher order reference materials:
http://www.bipm.org/en/committees/jc/jctlm/
Derivation of International Units:
http://www.nibsc.org/products/biological_reference_materials/frequently_asked_questions/how_are_international_units.aspx
Ordering standards from NIBSC:
http://www.nibsc.org/products/ordering_information/frequently_asked_questions.aspx
NIBSC Terms & Conditions:
http://www.nibsc.org/terms_and_conditions.aspx

12. CUSTOMER FEEDBACK

Customers are encouraged to provide feedback on the suitability or use of the material provided or other aspects of our service. Please send any comments to enquiries@nibsc.org

13. CITATION

In all publications, including data sheets, in which this material is referenced, it is important that the preparation's title, its status, the NIBSC code number, and the name and address of NIBSC are cited and cited correctly.

14. MATERIAL SAFETY SHEET

Physical and Chemical properties			
Physical appearance: White powder		Corrosive:	No
Stable:	Yes	Oxidising:	No
Hygroscopic:	No	Irritant:	No
Flammable:	No	Handling:	See caution, Section 2
Other (specify):	Contains inactivated influenza virus		

Toxicological properties	
Effects of inhalation:	Not established, avoid inhalation
Effects of ingestion:	Not established, avoid ingestion
Effects of skin absorption:	Not established, avoid contact with skin

Suggested First Aid	
Inhalation:	Seek medical advice
Ingestion:	Seek medical advice
Contact with eyes:	Wash with copious amounts of water. Seek medical advice
Contact with skin:	Wash thoroughly with water.

Action on Spillage and Method of Disposal

Spillage of contents should be taken up with absorbent material wetted with an appropriate disinfectant. Rinse area with an appropriate disinfectant followed by water.
Absorbent materials used to treat spillage should be treated as biological waste.

15. LIABILITY AND LOSS

In the event that this document is translated into another language, the English language version shall prevail in the event of any inconsistencies between the documents.
Unless expressly stated otherwise by NIBSC, NIBSC's Standard Terms and Conditions for the Supply of Materials (available at http://www.nibsc.org/About_Us/Terms_and_Conditions.aspx or upon request by the Recipient) ("Conditions") apply to the exclusion of all other terms and are hereby incorporated into this document by reference. The Recipient's attention is drawn in particular to the provisions of clause 11 of the Conditions.

16. INFORMATION FOR CUSTOMS USE ONLY

Country of origin for customs purposes*: United Kingdom * Defined as the country where the goods have been produced and/or sufficiently processed to be classed as originating from the country of supply, for example a change of state such as freeze-drying.
Net weight: 1g
Toxicity Statement: Non-toxic
Veterinary certificate or other statement if applicable**.** **Attached:** No